Genetic and environmental influences on tree nutrition in *Pinus radiata* D. Don

Impacts of nitrogen source in tree growth and root-associated communities

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A thesis submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
in Plant Biology
in the University of Canterbury

by

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To my whānau,

from Podes to Antipodes

Abstract

The main objective of the investigation presented here was to increase our understanding of how genotype-by-environment interactions influence nitrogen nutrition of pines. Nitrogen in soil ranges from simple inorganic forms (NH_4^+ and NO_3^-) to organic forms (amino acids, peptides and proteins), and microbially-mediated processes limit the availability of these molecules. Plants can only take up a portion of the N present in the soil solution, mainly in form of NH_4^+, NO_3^- and free amino acids. While an increasing number of studies have investigated species preference for different N forms and the resulting changes in plant development, the significance of intraspecific variation in plant resource use of organic and inorganic N forms and whether such variation translates to variation in root-associated communities, have not been established.

Growth studies in young radiata pine genotypes (*Pinus radiata* D. Don) showed that the amino acid L-arginine, alone or in combination with NO_3^-, can promote growth as effectively as, and sometimes even more effectively than, inorganic fertiliser (NH_4NO_3) at the same N concentration. The study was performed in both short-term greenhouse conditions (6 months) and longer-term field conditions (2.5 years), and results were confirmed in both systems. The study under field conditions revealed that the observed genotype variation to N source was further influenced by edaphic factors and interspecific competition with understory vegetation. In addition, root traits in three contrasting genotypes in response to organic and inorganic N form showed changes in the degree of root colonisation with ECM fungi and variation in root morphological traits, with no differences in a plant's capacity to take up any N source. In light of the significant impact of genotype and N form in growth and root traits, I then studied whether root-associated below-ground communities had a determining role in the observed variation.

In order to seek evidence of linkage between above and below-ground responses, I

investigated how the root microbiomes of two full-sib *P. radiata* genotypes with distinct physiological responses to organic versus inorganic N were altered in both greenhouse and field experiments. The results showed that intraspecific variation in tree response to N form, and the associated changes in soil physicochemical properties, have significant consequences for the root microbiome of *P. radiata*. The investigation showed that N supplied in either organic or inorganic form can differently influence plant and rhizosphere soil nutrient cycling, and these direct and indirect mechanisms can be associated with shifts in the diversity and composition of rhizophere microbial communities. Results from field conditions revealed that C:N stoichiometry of both rhizosphere soil and plant tissue decreased with the addition of L-arginine compared to the control, while adding an equivalent amount of N as NH_4NO_3 did not shift this ratio in either rhizosphere soil or tree needles. In addition, of the communities considered in the studies (rhizosphere bacteria, rhizosphere fungi, and root fungi) rhizosphere bacterial communities demonstrated genotype-specific responses to N treatment for both diversity and composition, measured in both the field and greenhouse. However, the effect of tree genotype and N treatments differently influenced rhizosphere and root-associated fungal communities, and this in turn differed between growth conditions, which suggests that edaphic and climatic factors evolve as strong filters of these communities.

Based on the results obtained in this study, it is clear that gene-by-environment interactions are important determinants of plant growth and physiological responses to organic and inorganic N source, and are strong determinants in shaping host-associated communities. This research suggests that, while subtle changes in genetic background can to some extent predispose trees to prefer one N source over the other, organisms comprising the root microbiota (including bacterial and fungal taxa) might be key determinants of soil N availability and plant capacity to access N sources. Therefore, the form of the N source differently influences above- and below-ground plant traits among genotypes of the same species, and these can affect the diversity and composition of the rhizosphere, either directly (by changing the nature of plant-microbe interactions) or indirectly (by altering soil properties in ways that favor particular microbial taxa).

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"A l'atzar agraeixo tres dons: haver nascut dona, de classe baixa i nació oprimida. I el tèrbol atzur de ser tres voltes rebel."

To fate I am grateful for three gifts: having been born a woman, of low class and oppressed nation. And the turbid azure of being three times a rebel.

- Maria Mercè Marçal, *Divisa*

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Chapter 1

General introduction

1.1 Natural and planted forests

The most recent analysis of forest data (FAO, 2015a) reports that total forest area covers 31% of the land surface of Earth, representing about 3.99 billion hectares. Approximately 30% of this area is distributed in boreal climate domains, 17% in temperate, 8% in subtropics and 44% in tropics. However, deforestation has decreased the global forest cover by 129 million hectares over the last 25 years (1990-2015). Trees and forests are of major importance to humankind, for economic, sociocultural and scenic services and values. Forests provide the basis of a variety of industries, including timber, processed wood and paper, and fruits that represent basic goods for a society. Wood is the primary energy source for about one-third of the world population and provides affordable shelter in addition to other environmental services that contribute to household welfare, especially for the people in many poor regions of the globe (FAO *et al.*, 2016). Many societies also have strong cultural attachments to their native forests, and depend on them economically, culturally and spiritually. In all these elements, the knowledge of the use of forest products, such as the vast diversity of medicines, foods, natural fertilisers and pesticides are critical for many communities and their co-existence with forests. Last but not least, the scenic and landscape services and values of forests involve ideas of aesthetics and beauty as components of forest ecosystem services.

Beyond the provision of goods and services for societies, forests and trees contribute strongly to a number of ecological services which are essential for ecosystem functioning at a global scale (Soussan *et al.*, 1995). Forest ecosystems have a determinant role in (i) the regulation of the hydrologic cycle, through the interception of rainfall and the control of its flow; (ii) the biogeochemical processes which maintain soil quality and the provision

of organic materials, through leaf/branch fall and root inputs; (iii) the contribution to biogeophysical processes, limiting erosion and protecting soils from the direct impact of rainfall and rivers; (iv) modulating climate; and (v) supporting and sheltering rich plant and animal biodiversity. The importance of forests in these roles has been exemplified in numerous studies which have observed and quantified changes that result from forest clearance (Sweeney *et al.*, 2004; Foley *et al.*, 2007).

The role of forests influencing climate has been long postulated, given their impact in biogeochemical and biogeophysical processes, despite the difficulties in making large-scale climate predictions (Bonan, 2008). Forest ecosystems store the majority of terrestrial carbon (approximateley 60%) mainly sequestered as organic carbon in the soil, but also in the vegetation biomass. Forests act as a carbon sink depending on the rate of C sequestration and this varies depending on factors such as forest age, biotic and abiotic stresses and human-induced deforestation (Streck & Scholz, 2006). However, it should also be noted that pine forests can cause losses of soil carbon, particularly in organic soils. In addition, forests control biogeophysical processes that influence climate through evapotranspiration and its role in the hydrologic cycle, by the formation of clouds and precipitation. It is well understood that maintaining forests globally would have a large impact mitigating the effects of anthropogenic greenhouse gases emissions on climate and associated factors (Bonan, 2008), which are key global changes. However, population and economic growth means that natural forest ecosystems and resources keep being degraded worldwide.

The increasing demand for raw materials and the dwindling of supply from natural forests has increased the importance of tree plantations. Planted forests for productivity represent 7.0% of total forest area - 277.9 million hectares - a 2.9% increase compared to 25 years ago (Payn *et al.*, 2015). The main difference between natural and planted forests is that plantations are created with the aim of growing highly productive forests on relatively small areas of land for more efficient land use, and are harvested after a set number of years. To maximise yield and economic return, trees require specific silvicultural practices in order to achieve their productivity potential. Regions with the largest areas of planted forests are East Asia and Europe, followed by North America, Southern and Southeast Asia (Payn *et al.*, 2015). Approximately 80% of species composition of the planted forests

comprise native species, while South America, Oceania and East and Southern Africa are regions dominated by planted forests with exotic or introduced species from outside the geographic region (Payn *et al.*, 2015). Although there is a wide variety of species used in plantation management, pines (members from plant genus *Pinus*) and eucalypts (members from plant genus *Eucalyptus*) present ideal characteristics and are used widely in planted forests, making up to 20% and 10% of the world plantations, respectively (Smith, 2001).

1.2 Factors influencing tree growth

The main abiotic environmental factors influencing tree growth, as for all terrestrial plants, are sunlight, water, soil and air temperature and nutrients from the soil. These are discussed in more detail below.

Light is a critical resource for plants that can limit growth and reproduction. As plants are sessile and photo-autotrophic, light is the sole energy source for photosynthesis. In the process of photosynthesis, the plant uses solar energy to oxidize water, thereby releasing oxygen, and to reduce carbon dioxide, thereby synthesising large carbon compounds, such as sugars (glucose). Plants normally compete for light, while leaves configure a canopy that absorbs light and influences photosynthetic rates and growth. For this reason, light energy also has an indirect effect regulating the plant form (Weiner *et al.*, 1990).

Water also plays a crucial role in the life of a plant. Photosynthesis requires the leaf absorption of carbon dioxide from the atmosphere, but this exposes plants to water loss. This leads to the dilemma that, of all the resources that plants need to grow and function, water is the most abundant and at the same time often the most limiting (Grace, 1996). Water availability may thus limit primary productivity in natural ecosystems, leading to marked differences in vegetation composition along precipitation gradients due to differences in drought tolerance (Stewart *et al.*, 1995). The proximate reason why this happens is that plants use water in large quantities, as a direct consequence of uptake of CO_2 for photosynthesis. Most water absorbed by plant roots is evaporated from leaves through transpiration, while only the 2% of the absorbed water is used for plant growth and other metabolic processes (Schulze & Hall, 1982). Water transport is passive and the spontaneous movement of water from regions of higher to lower levels results in the transport of water from the soil to replace the water lost by transpiration. Despite being a

passive transport, water loss is regulated by plants through stomatal opening and closing to minimise dehydration (Meinzer & Grantz, 1990). Factors such as the extension of the root system to extract water from the soil and the transport through xylem vessel elements and tracheids to bring water to the leaves, determine the efficiency of plant water use (Tyree & Zimmermann, 2002). Water movement is driven by the gradient of water potential, from high to low water potential. For example, during plant transpiration, opened stomata decreases leaf water potential and causes the movement of water toward the leaf surface. The regulation of water flow through the plant is mediated by small membrane proteins called aquaporins, which function as water channels and also maintain cell homoeostasis (Chaumont & Tyerman, 2014). Soil type and structure influence these processes and also influence water content, thus contributing to regulating the rate of water movement from soil to plants. For instance, sandy soils have lower surface area than clay soils, promoting water to drain though the pores more easily, making it less available for plant uptake.

Temperature limits plant growth and is a critical factor influencing the distribution of plants across different environments. Since photosynthesis is sensitive to temperature extremes, seasonal and diurnal temperature variations affect plants metabolism, thereby influencing the energy and resources necessary for growth, maintenance and reproduction (Yamori *et al.*, 2014). Given the lack of plants mobility, the capacity for plants to withstand and/or acclimate to unfavourable temperature in order for plants to survive. In addition, in trees changes in the optimum tree temperature can affect the rate of phenological development (Way & Oren, 2010), including bud set and dormancy. In many species, temperature changes together with daylight affect the autumn senescence and winter dormancy, affecting the length of the growing season (Rohde *et al.*, 2011). In flowering plants, this can also affect reproductive stages sensitive to high and cold temperature stresses, such as the reproductive duration, which can negatively affect plant productivity and crop yield (Zinn *et al.*, 2010).

Nutrients are chemical elements playing important roles in the metabolism of plants. Seventeen out of the 92 elements on earth are essential to all plants. Essential nutrients are classified as macronutrients and micronutrients depending on plant requirements. The macronutrients include nitrogen, potassium, magnesium, phosphorus, calcium, sulphur, carbon, hydrogen, oxygen and are usually present at > 1000 mg kg^{-1} - while C, H and O

make up approximately 95% of the plant biomass. The micronutrients include chlorine, iron, manganese, zinc, boron, copper, nickel and molybdenum and are typically present at < 100 mg kg^{-1}. In addition, the beneficial nutrients include aluminium, cobalt, sodium, silicon and selenium, which promote plant growth by partially replacing essential elements in a metabolic process (Subbarao *et al.*, 2003) and/or are essential for certain taxa under particular environmental conditions (Pilon-Smits *et al.*, 2009). Nutrients with greater requirement are nitrogen (N, 25 g kg^{-1} dry weight) and potassium (K, 10 g kg^{-1} dry weight) (Atwell *et al.*, 1999). Plants acquire nutrients through fine roots, which are found in the most distal parts of root systems. Plants take up nutrients dissolved in the soil solution, between soil particles, and this is generally enhanced by symbiotic interactions with mycorrhizal fungi (section 1.4). In the symbiotic relationship, plants benefit from improved water and nutrient uptake, while the fungus is provided with carbohydrates by the plant.

1.3 Nitrogen as a limiting growth factor

Whilst carbon dioxide and light are often present in excess, the availability of water and nutrients may limit growth. Nitrogen (N) in particular is often considered to be the most commonly limiting mineral nutrient to net primary productivity across terrestrial ecosystems (Vitousek & Howarth, 1991). Nitrogen is as an essential element in all organisms life cycle, also in plants, as it is needed to build important cellular components as RNA, DNA, proteins and amino acids, the building blocks of life. Amongst many other processes in plants, N is the major component of chlorophyll and the proteins in protein/chlorophyll complexes, and of enzymes in the carbon reactions of photosynthesis (e.g. Rubisco).

1.3.1 Terrestrial nitrogen cycle

Nitrogen is present in soils in various chemical forms, from complex organic forms - such as proteins - to simple inorganic ions - such as ammonium (NH_4^+) and nitrate (NO_3^-) - and can be either bonded to soil particles or dissolved in the soil solution. Plant-available N sources are usually present in the soil solution. Natural nitrogen budgets start with biological fixation atmospheric of N_2 to forms available to plants and microbes, such as

ammonium, through the action of free-living bacteria and symbiotic associations with plants (e.g. mainly *Rhizobium spp.* in leguminous plants). In addition, recent studies in pine species (Moyes *et al.*, 2016) provided evidence for N_2 fixation in *P. flexilis* by foliar endophytes, suggesting a potential new strategy for pines to acquire N thought associations with N_2-fixing bacteria. Fixation of N_2 also happens through the action of lightning may also contribute available N in ecosystems (Hill *et al.*, 1980). An important step in the terrestrial N cycle is the biosynthesis of nitrate by a series of biological processes, starting with the decomposition of soil organic matter (SOM) from plant litter and microbial biomass (Figure 1.1). Decomposition of plant litter in the top-soil is the second stage in which plant-available organic and inorganic N compounds are released into the soil solution. SOM is degraded to dissolved organic N (DON) through pH-dependent proteolytic activity (Weintraub & Schimel, 2005) and proteolytic enzymes from microbes exudation, mycorrhizal fungi and plant roots (Bajwa & Read, 1985; Abuzinadah & Read, 1986; Godlewski & Adamczyk, 2007; Paungfoo-Lonhienne *et al.*, 2008) in order to generate proteins and amino acids. Free amino acids are mineralisated (deaminated) by extracellular enzymes, releasing NH_4^+ and then taken up by plants and microbes (Figure 1.1). Ammonium can be further oxidised to nitrate or lost from the system by volatilisation. Nitrate that is neither taken up by plants nor leached is reduced in anaerobical conditions by denitrifying bacteria to atmospheric N_2, which closes the nitrogen cycle.

Plant yields in most agricultural and forest plantation systems are generally limited by N availability. Although it might be logical to assume that nitrogen-fixing microorganisms might have a competitive advantage against non-fixers, the extent to which energetic constraints or physiochemical stresses negatively impact nitrogen-fixing capacities under field conditions is not well known (Vitousek & Howarth, 1991). Production limitations by lack of N availability (section 1.3.2) have led to the use of the industrial conversion of atmospheric N_2 and H_2 to NH_3 by the Haber-Bosch process. This process was primarily developed (20th century) to supply the war industry with raw material for explosives, and revolutionised food production with the ability to manufacture mineral N-fertilisers (Smil, 2001). Since most of the N used in crops must be added to the environment in form of N fertilisers (Gruber & Galloway, 2008; Xu *et al.*, 2012), anthropogenic N inputs additionally contribute to the total N budgets in natural and managed ecosystems.

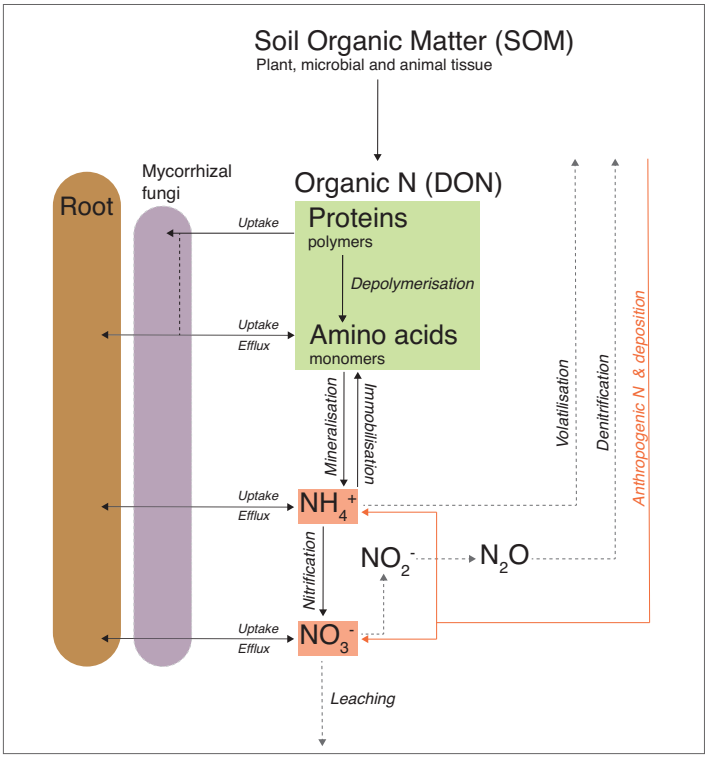

Figure 1.1: Simplified diagram of the terrestrial N cycle, that includes the main N pools of organic (green box) and inorganic (red boxes) N forms available for plants, either by direct uptake or through mycorrhizal transference. Dashed lines indicate N losses from the system by volatilisation or leaching, while red lines indicate anthropogenic N inputs and N deposition. The scheme does not include both animals and inputs via N_2 fixation.

1.3.2 Soil nitrogen availability

The availability of N sources depends on many ecological and environmental factors, such as the soil type and the contribution of various microbial-mediated processes. The two main mechanisms influencing N supply to root and fungal hyphae (for mycorrhizal roots) are mass flow and diffusion. When transpiration-mediated mass flow cannot move enough nutrients from the bulk soil to roots to meet plant N requirements, the supply of N to plant roots is mainly driven by the gradient of diffusion that develops around roots or hyphae as a result of active absorption of N compounds (Nye, 1980). This suggests that the relative abundance of organic and inorganic N sources is ultimately determined by the diffusive fluxes of each of the N compounds. Within those edaphic factors, the soil water content, the interaction of nutrients with soil colloids and the distance the nutrients must move to reach the root/hyphae surface are the main factors regulating this transport

mechanism (Barber, 1996). The latter two are strongly affected by the molecule size of the N chemical form.

There is a significant variability in the spatio-temporal distribution of inorganic N sources, with concentration ranges for NO_3^- and NH_4^+ between 100 μM and 10 mM (Xu *et al.*, 2012). In well-aerated soils, NO_3^- is usually more abundant than NH_4^+, although this might depend on factors such as the conversion rates by nitrifying bacteria (see Figure 1.1). In addition, NO_3^- has higher diffusion coefficient, making it more accessible for plant uptake (Miller & Cramer, 2004). However, because NO_3^- in soils is heterogeneously distributed in time and space and often confined in soil N patches, NH_4^+ might be more available in climates with low temperatures, low pH and high soil water content, given the limitation of microbial transformations of NH_4^+ (Kronzucker *et al.*, 1997). Furthermore, it has been proposed (Kronzucker *et al.*, 2003) that temporal variations driven by the ecological succession of ecosystems significantly influences the availability of NO_3^- and NH_4^+, with NO_3^- often dominating the early-successional stages of ecosystem development, and NH_4^+ increasing in abundance as succession progresses.

Amino acids in soils are present as (i) free amino acids, (ii) exchangeable forms bound to soil particles and soil organic matter and (iii) part of proteins and peptides, with the latter being the most commonly found. Plants absorb organic N mainly in form of free amino acids, though can take up small peptides and proteins (Paungfoo-Lonhienne *et al.*, 2008; Soper *et al.*, 2011). It is difficult to find consensus about the relative concentrations of free amino acids in soil, with concentrations ranging from 0 to 100 μM (Monreal & McGill, 1985; Owen & Jones, 2001). Studies generally report that free amino acids represent the largest fraction of low molecular weight dissolved organic N (LMW-DON) (Jones *et al.*, 2005a) but account for the smallest fraction of DON - and both peptide- and protein-bound amino acids contribute more than the half of the DON pool. Depending on the charge of the side chain, amino acids can be grouped as acidic, neutral and positive at pH 7. This structural fact differentially affects amino acid mobility at a particular pH and influences the molecule's diffusion rate (Miller & Cramer, 2004). In addition, soil is a highly dynamic and heterogeneous environment, and the relative abundance of amino acids is not static and results from synthesis (organic matter degradation, cells release) and consumption/interconversion processes (mineralisation and assimilation) (Moe, 2013).

Nonetheless, the methodologies used to analyse the relative abundance of each of the 20 proteinogenic amino acids are as diverse as the concentrations found (Jones *et al.*, 2005b; Inselsbacher *et al.*, 2011). Inselsbacher *et al.* (2011) conducted a comparative analysis of soil extraction and passive diffusion sampling (microdyalisis techniques) and revealed that the former measured the highest concentrations of ammonium and nitrate, while the latter showed plant-available amino acids contributed greatly to the total N pool over time. This lack of consistent information on available plant N chemical forms emphasises the technical challenges in reaching high spatio-temporal resolution for soil N chemistry analysis (Inselsbacher, 2014; Inselsbacher *et al.*, 2014).

Given the increase in the appreciation of amino acids as key intermediates in the soil nitrogen cycle, a new conceptual model of the limiting steps in the soil N cycle has recently emerged (Schimel & Bennett, 2004). According to this, N-limitation is due to the depolymerisation rates of proteins to peptides and amino acids rather than mineralisation rates. This emerging theory is based on the evidence that plants, in order to meet their requirements, can acquire, and even sometimes entirely rely on organic N sources (rather than NO_3^- and NH_4^+), in order to meet annual plant requirements. This renewed vision of the N cycle represents an alternative to the traditional view in which mineralisation and associated environmental factors (i.e. soil temperature, pH, soil moisture) exclusively determine N availability for plant root uptake.

1.3.3 Plant acquisition and utilisation of nitrogenous compounds

Plant N nutrition or metabolism can be seen as the integration between the uptake and utilisation of accessible N sources in the environment. The interaction of multiple environmental and genetic factors influence the rate and efficiency of plant nutritional mechanisms. When these meet plant N demands, biomass production in above- and belowground occurs. The key steps in N metabolism are uptake and utilisation, with the latter being further divided into assimilation, translocation and remobilisation.

Because of the constraint of evolving in naturally N-limiting soils, some plants have developed regulatory mechanisms to control the main steps in N metabolism and utilisation in order to guarantee the economy of this essential nutrient. Besides the N demand for aboveground growth, plant development modulates the root acquisition and utilisation of

the available resources - e.g. fructification (Rennenberg & Gessler, 1999), leaf and roots development (Silla & Escudero, 2003). In addition, the plants environment may change during their lifetime. Plants are highly complex systems and hold multiple signals that enable them to adjust functions, including N metabolism, to these environmental changes - e.g. in response to salinity (Gimeno *et al.*, 2009) or drought (Rennenberg *et al.*, 2006).

N uptake efficiency is defined as the percentage of N acquired at a given N concentration relative to plant biomass, the so-called nitrogen uptake efficiency (Xu *et al.*, 2012). This is experimentally determined using stable isotopes, by measuring the total ^{15}N incorporated after ^{15}N enrichment with single (^{15}N-N) or double (^{15}N-^{13}C) N-labelled compounds, depending on whether the N sources studied are inorganic or organic, respectively (Näsholm *et al.*, 1998). This technique allow the study of the rates of N acquisition by plant roots over time, either under controlled or realistic growth conditions.

The potential of plant roots to absorb N from the soil primarily depends on (i) soil N availability (section 1.3.2), (ii) the affinity and regulation of N-uptake transporters (sections 1.3.3.1 and 1.3.3.2), (iii) the internal N status of the plant (section 1.3.4) and (iv) the root structure which determines the capacity of roots to physically intercept nutrient (i.e. root architecture, root plasticity) (section 1.5.1). Based on these factors, plant species show phenotypic plasticity in response to different N forms, but may also show a preference for uptake of organic and inorganic N forms (Weigelt *et al.*, 2005). Preference for a particular N form can be defined as the enhanced uptake or the increased growth when that N form is supplied (Britto & Kronzucker, 2013). These issues are discussed in the following sections.

1.3.3.1 Root uptake and assimilation of inorganic nitrogen

The uptake of inorganic N forms (NO_3^- and NH_4^+) is well characterised. This area of research, has contributed to the identification of genes encoding transport membrane proteins directly involved in the uptake, as well as the regulators of the inorganic N uptake (reviewed by Miller & Cramer, 2004; Glass, 2009). The transport systems responsible for the uptake of NO_3^- and NH_4^+ are the High and Low Affinity Transport Systems, referred to as HATS and LATS respectively, both dependent on energy from adenosine-5-triphosphate (ATP), and induced at low and high N concentrations, respectively. In the case of nitrate

acquisition, HATS are either constitutively expressed or induced by NO_3^-. HATS and LATS are transcriptionally and post-transcriptionally regulated by their respective substrate and the expression of both transporters is controlled by downstream metabolites and glutamine (Gln), and also regulated by light (reviewed by Glass, 2009).

After uptake, NO_3^- can be stored in the vacuoles or assimilated into carbon (C)-skeletons to form amino acids. In the latter process, NO_3^- is first reduced to ammonium (NH_4^+) and then incorporated into organic forms ready for transportation within the plant. When N availability increases, it stimulates the synthesis of amino acids and proteins, otherwise the NH_4^+ excess can promote the formation of toxic amides (Krajina *et al.*, 1973). Ammonium may be generated by direct primary NO_3^- or NH_4^+ assimilation, or from secondary metabolism such as photorespiration (Coruzzi, 2003). The initial reduction of nitrate (NO_3^-) to nitrite (NO_2^-) occurs in the cytoplasm by the nitrate reductase enzyme (NR). Nitrite (NO_2^-) is converted to NH_4^+ in the plastid or in the chloroplast depending on the form of the nitrite reductase enzyme (Foyer *et al.*, 2011). Then, NH_4^+ is rapidly assimilated into C skeletons to produce amino acids by the glutamine synthetase/glutamate-2-oxoglutarate aminotransferase (GS/GOGAT) enzymatic pathway (in the mitochondria, chloroplast and cytosol), regardless of whether it is derived from NO_3^- or directly taken up from soil. The end products of this cycle, glutamine and glutamate, are the N donors for the biosynthesis of major N compounds, such as amino acids that play key role in mobilisation/recycling N depending on the demand. The synthesis of these N compounds involves the action of a wide range of multispecific aminotransferase and nucleic acid bases, polyamines and clorophylls. Overall, the regulation of uptake and assimilation of NH_4^+ and NO_3^- is under the strict control of a complex network of signals originating from C- and N-metabolism as well as from plant hormones (Rubio *et al.*, 2009).

In forest ecosystems, trees have become specialised to absorb mineral nitrogenous compounds that are most available in their native environment (Kronzucker *et al.*, 1997), and a number of studies have demonstrated that small trees and specifically, conifer seedlings, show low uptake rates of NO_3^- (Öhlund & Näsholm, 2001; Miller & Hawkins, 2007). Such specialisations may have evolved as a competitive strategy that might depend on the successional stages of the ecosystems, which ultimately determines the levels of nitrification and the NO_3^- / NH_4^+ availability. For example, late-successional coniferous

species, such as *Picea glauca*, have shown superior growth and N uptake when growing under NH_4^+ compared to NO_3^-, and this has been associated with a poor utilisation of NO_3^- at the level of uptake, metabolism and intracellular storage (Kronzucker *et al.*, 1997). However, after disturbances such as fire, avalanche clearing, windthrow and clearcut harvesting (Vitousek *et al.*, 1982), ecosystems may become NO_3^--rich, and this promote the establishment of early-successional species that prefer NO_3^- over NH_4^+ (Malhi *et al.*, 1988).

1.3.3.2 Root uptake and assimilation of organic nitrogen

The majority of mycorrhizal and non-mycorrhizal plant species studied so far have been found to be capable of the uptake of organic forms (amino acids) and utilise such compounds for growth, across diverse experimental conditions (reviewed by Näsholm *et al.*, 2009). Plants possess different families of amino acid transporters and the uptake of amino acids is an active process powered by the proton gradient across the plasma membrane in root cells (Rentsch *et al.*, 2007). These transporters may participate in the uptake of amino acids from the soil solution as well as the recovery of amino acids that have been released by efflux. In *Arabidopsis*, the lysine-hystidine transporter 1 (LHT1) - from to the amino acid transporters (ATF) superfamily - and amino acid permease 5 transporter (AAP5) - from the amino acid-polyamine-choline (APC) superfamily - have been recently functionally characterised (Svennerstam *et al.*, 2011). The function of such transporters involved in amino acid uptake provides evidence that plants can bypass the N mineralisation step by absorbing intact amino acids. In addition, the co-expression of such transporters in the root surface (i.e. redundancy) suggest there are different environmental developmental signals that may regulate the uptake of amino acids (Liu & Bush, 2006).

Very few studies have addressed the metabolism of amino acids following their root uptake. However, it is plausible that after amino acids enter the plant N pool they might be directly used for protein synthesis, deaminated or transported to sink tissues (Miller & Cramer, 2004). Arginine is considered to be an ideal nitrogen storage form, because of its nitrogen-rich structure that minimises the osmotic impact (Edfast *et al.*, 1996). In trees, amino acids are the currency of nitrogen exchange between source and sink tissues, and it is essential that there is a long-distance transport systems delivering the amino acids to the sink organs. In pines, and in particular *Pinus taeda*, over 46% of total N stored in the

megagametophyte reserve is arginine, and represents the 23% of the plant amino acid pool (King & Gifford, 1997). There is evidence that the complex NAGK-PII, responsible for the inhibition of arginine synthesis, might be a highly sophisticated activation mechanism that renders possible massive nitrogen storage as arginine (Llácer *et al.*, 2008). When nitrogen is scarce, arginine is catabolised to ornintine and urea. Following this, ornithine is metabolised to other amino acids, while urea dissociates to NH_4^+, which is reassimilated by the GS/GOGAT enzymatic pathway (Cánovas *et al.*, 2007).

Despite the broad evidence that plants have a functional capacity to uptake and metabolise amino acids, and the fact it has been more than one century since that the capacity of plants to absorb amino acids was first postulated (i.e. Read, 1991; Lipson & Näsholm, 2001; Schimel & Bennett, 2004), the ecological significance of amino acids as N sources for plant nutrition is still questioned (but see Ganeteg *et al.*, 2017). While the preferential use of organic N in cold and N-limited environments has become increasingly accepted, the contribution of free amino acids to tree N budget in temperate forest soils has only recently been considered (Rothstein, 2009). At least three major key points have been the topic of intense debates. First, some authors argue that plant's capacity to take up reduced organic N forms is restricted to the role of mycorrhizal fungi (Read & Perez-Moreno, 2003) (section 1.4.1). Second, many authors have provided evidence that plants cannot take up significant amounts of amino acids since they are poorer competitors for organic N compared to soil microbes (Harrison *et al.*, 2008) (section 1.4). Third, evidence for the uptake of amino acids as intact molecules using the dual C/N labelling technique has been debated. Some studies using dual-label organic N-sources sometimes lack the ^{13}C enrichment in the plant tissues which is required to confirm direct uptake (Hodge *et al.*, 2000). This last point has been claimed to be the result of the rapid assimilation of absorbed organic N compounds by plant roots, so part of the carbon acquired might be lost through respiration (Näsholm *et al.*, 2009).

1.3.4 Nitrogen metabolism and allocation of plant biomass

Plant growth and primary production depend on the plant N content. Nitrogen content in plants is related to carbohydrate production and dynamics, given the essential role of N in photosynthesis. Generally, changes in C and N economies and in the carbon-

to-nitrogen ratio (C:N) are key determinants in variation of plant growth rate. Brouwer (1962) suggested that the internal balance between C and N in plants is the driving force of biomass distribution between plant organs. This is based on the assumption that nutrients (i.e. N) are acquired by roots while C is fixed by the shoot (i.e. leaves), and that source tissues (i.e. roots, shoots) are primary receivers of the resources acquired (i.e. N, C). Biomass allocation or distribution is defined as the relationship of one organ biomass to that of another. For example, in response to limited N availability, plants generally allocate more biomass to roots than shoots, in order to increase the allocation of resources to the source rather than the sink tissues (i.e. root absorptive area).

In order to account for differences in shoot and root allocation and to mathematically describe such differences, it is widely accepted that the use of the root-to-shoot ratio (root:shoot) is very informative on the supply, transport and utilisation of N and C by roots and shoots. However, the use of root:shoot is restricted to a certain point in time. This is problematic because the descriptor inherently changes depending on the stage of plant development (ontogenetic drift; i.e generally greater at early stages of development) and plant-size. For example, comparative studies using different species with different growth rate suggest that fast growing species have greater nitrogen content and allocate greater nitrogen to leaves, with higher rate of photosynthesis per unit of N in the leaves than that in slow growing species (Poorter *et al.*, 1990).

Few studies have explored the effects of inorganic N sources (i.e. NO_3^- and NH_4^+) in biomass distribution in trees and have presented inconsistent results (Bauer & Berntson, 2001; Bown *et al.*, 2010). They often suggest that N concentration used, but not the form of N, affects biomass allocation over time. In contrast, recent studies of biomass partitioning in plants growing in equimolar concentrations of organic and inorganic N have provided evidence that plants growing on amino acids might influence root morphology and increase root:shoot (Cambui *et al.*, 2011), although others indicate it might not (Wilkinson *et al.*, 2015). These results may reflect differences among growth conditions, plant species and other associated abiotic and biotic factors which interact with the biochemical effects of N form. Broadly in line with this, some authors have recently postulated that lower metabolic costs of assimilation for organic N than inorganic N can influence overall growth and biomass distribution at longer term (Gruffman *et al.*, 2013; Franklin *et al.*, 2017).

Conversely, others have claimed that this metabolic advantage might be overestimated due to greater investment of resources required for organic N uptake (Zerihun *et al.*, 1998).

1.4 The role of rhizosphere organisms

The rhizosphere is a narrow zone (few mm thick) immediately surrounding the root area, comprising soil, microbial communities and root-secreted compounds. Soil in the rhizosphere is between 10- to 100-fold richer in microbes than bulk soil (soil outside the rhizosphere) (Lugtenberg, 2015) because of the greater abundance of plant-derived C released by roots (approximately 11% of the total C fixed) (Jones *et al.*, 2009a). Microbes in the rhizosphere are grouped into the so-called *rhizosphere microbiome*. The rhizosphere is an extremely complex and dynamic interface, where many different interactions between roots, microbes and soil occur. Over the past decade, a number of new methodologies have emerged, from high-throughput next-generation sequencing techniques - such as metagenomics and metabarcoding - to stable-isotope probing that increase our understanding of species and processes that operate in the rhizosphere. Considerable progress has been made showing that the belowground system - integrated by root structures and associated communities - influences aboveground plant growth and that root-associated communities play a major part in plant adaptation to biotic and abiotic stresses (Wardle *et al.*, 2004; Berendsen *et al.*, 2012). Although the ecological and evolutionary factors shaping the rhizosphere microbiome are still unclear, some authors consider the rhizosphere microbiome as a *secondary genome* that provides host plants with favourable microbe-derived compounds and traits or functions that improve adaptability and productivity (Berendsen *et al.*, 2012). Moreover, novel insights into the significance of such interactions in biogeochemical cycling, soil formation and Earth history (Lambers *et al.*, 2009) suggest that the dynamics of the root-microbe interface should be included in larger scale models describing biosphere processes and their responses to global change drivers. Numerous organisms contribute to the rhizosphere microbiota, including bacteria, fungi and archaea that feed from rhizodeposits, leading to a wide variety of interactions between plants, antagonists and mutualistic symbionts (Lugtenberg, 2015).

Evolution has adapted soil microbiota to assemble into specific niche conditions,

characterised by specificities based on the plant community diversity and soil environments (Tewari & Arora, 2013). Soils are complex and heterogeneous in their properties - i.e. pH, structure, texture and organic matter content. Recent studies have demonstrated that the physico-chemical characteristics of soils select for specific microbes by creating niche environments that recruit certain types of microbes (Lundberg *et al.*, 2012). Soil properties also influence the rate of release and composition of root exudates, which in turn directly promote microbial selection by plants. The relative importance of the multiple biotic and abiotic factors that influence this multidimensional system (soil-microbe-roots), varies depending on the ecosystem (i.e. natural or managed) and the stage in ecosystem succession (i.e. primary, secondary succession) (Lambers *et al.*, 2009). Due to co-evolution, plant species in natural ecosystems are expected to benefit to a greater extent from rhizosphere microbiota, which should lead to stronger positive feedback on plant performance than in agricultural ecosystems (Philippot *et al.*, 2013). Agricultural crops grow in many different soil types, and given the use of fertilisers and pesticides, plant growth and health are able to rely less on the rhizosphere biota, thus invoking a greater potential impact of soil type. Interestingly, it has also been postulated that plant breeding might have unintentionally selected against plant traits that are essential for hosting and supporting beneficial microorganisms (Germida & Siciliano, 2001). As introduced before, ecosystem succession strongly drives plant and microbial community diversities due to variation in soil resource availability. In young soils, a steady increase in soil N content occurs, and over time this results in a progressive change from a predominantly N-limited to increasingly phosphorus-limited communities (Parfitt *et al.*, 2005). For example, poorly developed young ecosystem with depleted nutrients favour plant associations with N_2-fixing symbionts, which live at the expense of the C fixed by the plant (Sprent, 2007).

Roots have been shown to be selective of rhizosphere microbial populations (Hartmann *et al.*, 2009). The rhizodeposition of plants is composed of nutrients, exudates, border cells and mucilage released by roots that promote community shifts in the rhizosphere. Root exudation can stimulate specific microbial communities through the release of (i) nutrients and energy sources (i.e. C compounds) for heterotrophic microorganisms, (ii) compounds such as carboxylates and phenols that contribute to the mobilisation of insoluble minerals and, (iii) antimicrobial compounds that inhibit or discriminate against

particular microbial populations. Root exudation can accelerate SOM decomposition by the release of labile C compounds that contribute to microbial growth (Dijkstra *et al.*, 2013). In contrast, the release of recalcitrant C-rich exudates causes the immobilisation of nutrients to an extent that inhibits the activity of saprotrophs, so decomposition slows (Lindahl *et al.*, 2010). Other more complex molecules, such as flavonoids, strigolactones and sesquiterpenes have been identified as signalling molecules that are important for regulating the communication between plants and rhizosphere biota (Akiyama *et al.*, 2005; Hassan & Mathesius, 2012). Plants can also apply other less-specific strategies to select rhizosphere microbial communities, such as the alteration of pH and the redox-milieu in the rhizosphere (Blossfeld *et al.*, 2011).

Plant species, genotypes and *cultivars* impact the diversity and composition of the rhizosphere microbiome (e.g. Schweitzer *et al.*, 2008a). Interactions between plant identity and rhizosphere biota are not a new concept. Traditionally, biological processes in the rhizosphere were considered to be net outcomes of antagonistic and mutualistic relationships, and research efforts focused on the effect of pathogens, nitrogen-fixing microbial symbionts and mycorrhizal fungi (Lareen *et al.*, 2016). However, recent studies have indicated that plants rely on and actively recruit a range of soil microorganisms in the rhizosphere (Lau & Lennon, 2012). Aside from the effects of specific pathogens and symbionts on plant health, the composition of the rhizosphere microbiome can have significant impacts both on plant development and stress tolerance (Panke-Buisse *et al.*, 2015).

1.4.1 Microbiome-associated plant nitrogen responses

A large number of studies have suggested that microbiota in the rhizosphere can influence the way plants access soil N sources mainly by (i) competing for the rhizosphere availabile N or (ii) altering the capacity of plants to acquire N sources. The contribution of these two processes in plant growth has been mainly supported in the context of N-limited ecosystems. In such conditions, the depletion of N sources might be critical for the co-existence of micro- and macroorganisms (Harrison *et al.*, 2007), but might also select for those plant competitive strategies that rely on beneficial relationships with symbiotic fungi to expand the available resources (Brzostek *et al.*, 2015). In a context where the frequency and intensity of extreme climate events affecting the N cycle (i.e. N deposition)

keep increasing, it is important to better characterise and understand the capital, yet poorly understood, role of plant/microbe interactions in biogeochemical processes. This must be the subject of further research.

The increasing evidence that plants can take up and use intact organic N sources, has led to a re-evaluation of plant-microbe competition for N, including both spatial and temporal aspects of N availability (reviewed in Kuzyakov & Xu, 2013). As discussed above (section 1.3.3), the degree to which free amino acids in the soil solution contribute to the overall N budget of a plant is currently not well quantified. However, the rate of organic N mineralisation and the competition with soil microorganisms are both likely to be important drivers. There is strong evidence to support the idea that plant roots might have a poor competitive ability to capture amino acids from the soil (Hodge *et al.*, 2000; Owen & Jones, 2001). Other authors have hypothesised that a plant's capacity to take up organic N uptake represents an evolutionary adaptation against the pressure of environments with low availability and diversity of N sources (McKane *et al.*, 2002; Kahmen *et al.*, 2006). Consequently, due to niche exclusion, plants that naturally grow in such ecosystems can successfully compete with microorganisms for the available N sources. For example, in the Arctic tundra, N acquisition from mineralisation may account for less than 50% of total uptake, which suggest that plants may use amino acids in addition to other organic N forms (Kielland, 1994; Schimel & Chapin, 1996). The same may occur in boreal forests, dominated by *Pinus sylvestris*, where there is a low rate of mineralization, and amino acids are the dominant N source (Lipson & Näsholm, 2001).

The majority of tree species have roots that are colonised by ectomycorrhizal (ECM) and arbuscular (AM) fungi. From the plant perspective, this association functions as extensions of roots and facilitates plant nutrient and water uptake. By increasing root surface area and metabolic profile, ECM and AM facilitate the extraction of nutrients that could not be reached by roots alone (Read & Perez-Moreno, 2003). Coniferous roots are greatly colonised by ECM fungi, which form a thick mantle beyond the root zone. There are at least 20,000-25,000 ECM fungal species known to associate with plants (Rinaldi *et al.*, 2008), and these vary greatly (both between species and among strains) in their ability to access nutrients (Hodge *et al.*, 1995). Ectomycorrhizal fungi have the capacity to take up different N sources, either organic and inorganic N (Abuzinadah & Read, 1988; Talbot &

Treseder, 2010) but exhibit a preferential uptake for amino acids, which present reduced cost compared to inorganic N (Allen *et al.*, 2003), and rarely participate in the nitrification of ammonium. This might have important negative implications for nitrate leaching due to the N enrichment that occurs after the anthropogenic N deposition in ECM-dominated forests. More importantly, the influence of mycorrhizal traits on ecosystem C cycling has been recently investigated using a novel modelling approach (Orwin *et al.*, 2011). The study provides evidence of a significant increase in soil C storage as a consequence of the direct uptake of organic nutrients by mycorrhizal fungi, with a potential role on the ecosystem functions.

In the recent years, the ECM fungal model *Hebeloma clyndosporum* contributed greatly to improve our understanding of N uptake and assimilation. This includes the identification of transporters mediating the uptake of nitrate, ammonium and amino acids but also the putative export of organic and inorganic N forms to root cells in *Pinus pinaster* (Müller *et al.*, 2007). However, is still uncertain whether symbiotic nutrient transport processes are analogous to processes for non-mycorrizal partners, or whether new root transporters are expressed as a result of the interactions.

Beyond the well-known positive effects of mycorrhizal fungi in plant growth and development, the diversity of interactions between plants and mycorrhizal species contribute more broadly to a wide variety of relationship types across the mutualism-parasitism continuum (Karst *et al.*, 2008). The degree to which mycorrhizal fungi are mutualistic and beneficial for the plant host may depend on complex interactions between (i) the amounts of carbohydrate allocation from the host (i.e. amount of C transferred), (ii) cost-benefit for the mycorrhizal fungi, and whether nutrient scavenging is more effective than the exchange with the host plant, (iii) plant community composition, even at the genotype level, and (iv) fungal community composition (Smith & Read, 2008; Lamit *et al.*, 2016). For example, greater allocation of photosynthates would attract fast-growing mycorrhizal fungi, which are characterised by greater investment in external hyphae than colonisation (Treseder, 2005). This type of mycorrhizal association is more efficient in scavenging nutrients than transferring them to the host, and consequently would retain or immobilise nutrients for its own biomass growth rather than transferring the nitrogen to plants (Karst *et al.*, 2008). This spectrum of relationships is likely to be also strongly affected by the

availability of N in the environment and the preference or capacity of mycorrhizal types for the uptake of certain N sources.

1.5 Intraspecific genetic variation

1.5.1 Phenotypic plasticity

Phenotypic plasticity is a measurable variation in form and/or function and is defined as the capacity of a single genotype to exhibit a range of phenotypes in response to variation in the physical, chemical and/or biotic characteristics of the environment (Fordyce, 2006). A current challenge in biology is to understand the underlying cause of developmental plasticity and clarify the extent to which biological traits are manifested as genotypic variation from evolutionary/breeding history (G) (proportion of phenotypic variation attributable to genes), as a phenotypic response to environmental variability (E) (proportion of phenotypic variation caused by the environment) or as the genotype-by-environment interaction (G x E). The latter is defined as both, (1) the causal relationships between the environmental factors and the responses of the various traits defined by the genetic background and (2) the traits influenced by many different genes and by the multitude of environmental conditions at different stages during plant development (Whitman & Agrawal, 2009). The genotype-by-environment interaction is apparent when different genotype of the same species respond differently to the same environmental conditions, in what is called intraspecific genetic variation. Whether the expression of genotype-specific traits extends to other environments will depend on the genotype fitness or performance (flexibility) and to the biotic/abiotic factors influencing the new conditions.

Historically, plasticity was considered as noise confounding the study of true genetic determinants through the organism's development. However, more recently, it has been recognised as an important means to develop function in response to the environment (Sultan, 2000). Plasticity exists in both plants and animals, but has been most intensely studied for plants, because they have a sessile nature and continue organogenesis throughout life (Bradshaw, 1965). Animals seem to promote developmental plasticity during adult life, while plants renew stem cell populations to continuously form all post-embryonic organs. There are several examples of plant phenotypic responses, such as the developmental

responses to avoid shape damage (Aphalo *et al.*, 1999) and the alteration of root allocation and architecture in response to varying soil conditions (Bell & Sultan, 1999). There is an important need to increase our understanding of how plant genotypes respond differently across environmental gradients, for evolution, ecological and agronomic purposes.

1.5.2 Importance of genotype-by-environment interactions

Many studies have examined phenotypic plasticity as the way for a plant to adapt to its environment as an individual (reviewed by Bradshaw, 1965), based on the natural development of mechanisms leading to tolerance, resistance and avoidance of environmental constraints. Although plasticity is not required to be beneficial or to have undergone adaptive evolution, it is often associated with evolution. Plasticity evolution is very complex. It is based on how individuals interact with the environment. It presumably integrates the benefits of plastic changes in different environments, the ecological trade-offs (i.e. fitness, lifespan vs. stress period), ecological feedbacks (i.e. when phenotypes change the environment, and this affects selection of other organisms) and plasticity interactions between genomes (Valladares *et al.*, 2007; Nicotra *et al.*, 2010; Aspinwall *et al.*, 2015). The importance of plasticity has been often associated with the individual's lifespan. Importantly, long-living sessile terrestrial organisms, such as tree species and populations, come with very long generation times and the ability to tolerate a wide range of edaphic and climatic growing conditions, ranging from the northern subarctic region to subtropical zones. For example, conifers display wide ranges of environmental heterogeneity, making them *experts* in adaptation. Trees and perennial woody plants from the temperate and boreal regions have developed systems to modify their phenotype to tolerate changes in climatic conditions. Moreover, it is important to emphasise that processes of adaptation throughout an individual's development are adaptive phenomena that do not always fit well into the traditional Mendelian genetic framework (based on changes in allelic frequencies), and authors suggest they might be shaped by epigenetic mechanisms (Yakovlev *et al.*, 2011).

Recent studies have proposed that plant phenotypic or genotypic variation in particular ecosystems have far-reaching impacts on nutrient availability (Treseder & Vitousek, 2001; Hodge, 2004; Fischer *et al.*, 2017), belowground productivity (Fischer *et al.*, 2007), and plant water-use (Fischer *et al.*, 2004). Interestingly, climate change is potentially

altering conditions and the availability of resources, and in novel conditions, plants must either adapt or migrate to seek conditions for which they are adapted (reviewed by Nicotra *et al.*, 2010). The study of molecular and genetic mechanisms underlying plasticity is particularly relevant to the climate change scenario, and critical to understand and predict environmentally-induced shifts in plant phenotypes. In the recent years, an attempt has been made to apply in crop systems the ecological and evolutionary theory regarding plasticity. Traditionally, research in crop science and modern breeding have been focused on selecting plant traits to obtain high yield in particular environments (Tester & Langridge, 2010). However, it is unclear whether domestication and breeding have increased or decreased plasticity in traits associated with yield. It seems logical though, that breeding for phenotypic plasticity in traits other than yield will improve adaptation though the adoption of adaptive strategies to the increasingly unpredictable environment (Nicotra *et al.*, 2010). For example, breeding for phenotype plasticity in traits related to the efficiency of nitrogen use efficiency (NUE) or water use efficiency (WUE) could lead to better survival and higher average yields. NUE is a heritable trait which has also been demonstrated to be sensitive to the environment (Burdon, 1976; Knight, 1978). However, NUE is also a complex genetically controlled trait depending on phenotypic plasticity through both N-regulated root architecture and N transporters, so the modification of a single transgene (i.e. GMO) would be inadequate to achieve a significant benefit. Apart from improvements in production, NUE has additional implications for the long-term management of ecosystems towards more sustainable practices (Hawkins *et al.*, 2010). We must consider the challenge of improving productivity with fewer inputs through an understanding of the underlying mechanisms contributing to phenotypic plasticity (Valladares *et al.*, 2007).

The ability to alter root systems in order to maintain function and growth in soils with low availability of resources is another aspect of a plant's individual adaptive plasticity (Grime, 1994). Plant root systems have developed increasing complexity since early ancestors to explore the soil more effectively, acquiring the ability to sense and respond to a range of environmental cues (Porterfield, 2002). The main abiotic factors or stimuli influencing root development are (i) temperature, (ii) water availability, and (iii) nitrogen and (iv) phosphorous availabilities (McCormack & Guo, 2014). The capacity to convert variation in these stimuli (e.g. accumulation or depletion of limiting nutrients in soil) to

developmental responses is determined by the level of turnover and morphological plasticity in root system architecture. In general, an increase in plant productivity contributes to the increase in the root carbohydrates reservoir, which promotes root turnover. In contrast, colonisation by mycorrhizal fungi and root endophytes increases root lifespan and benefits growth due to the provision of nutrients and water. Ecologically, the inherently plastic nature of woody tree root systems compared with herbaceous ones (Schenk & Jackson, 2002) has enabled trees not only to access water and nutrients for their own benefit, but also to redistribute water resources and remobilise nutrients. Therefore, the implications of plasticity in root structures of tree species are not only to ensure their own survival over inter-seasonal changes over long-time periods, but also to maintain the dynamics of biogeochemical cycles and associated ecosystem services (Clemmensen *et al.*, 2014).

1.5.3 Extended phenotype

A few decades ago, Richard Dawkins popularised the biological concept of *the extended phenotype*, in which he proposed that phenotypes are not limited to the biological processes expressed in the individual genes, but also extended to include all effects that a gene has on its environment, either inside or outside the individual organism (Dawkins, 1982). Recent studies support the importance of phenotypic plasticity in shaping communities, so that heritable traits in a single species (i.e. genotype variation) has community and ecosystem consequences. The extended phenotype originates from the individuals possessing the trait, and then extends to the community, and ultimately to ecosystem processes (Whitham *et al.*, 2003). Recent studies have shown that plants and their associated organisms are highly responsive to their environment. As such, interactions between genotype and environment (G x E) are of key importance, contributing to the predictability of associated community structures and ecosystem processes based on genotype-specific traits (Whitham *et al.*, 2006).

Although there are many studies exemplifying these kinds of interaction, it is assumed that most of them are unknown. For instance, Whitham *et al.* (2008) showed that variation in the associated community composition of arthropods within the canopies of *Populus* trees was genetically determined by the content of condensed tannins in leaves.

Similarly, recent studies (Lamit *et al.*, 2016) also reported the covariance between senescent leaf chemistry and ECM fungal composition, providing further evidence that above- and belowground linkages can have important implications in the interactions between host genotype and the associated communities. Further research has also linked the effect of genotype host with coexisting microbial communities in both root and leaves (Wagner *et al.*, 2016), suggesting that the extension of genotype variation in associated communities also integrates interactions within the community that are simultaneously responding to the host genotype. Extensive work on the genetics of tree productivity and recent work on genotype-by-environment interactions in forests (reviewed by Whitham *et al.*, 2012) suggest that plant productivity may be a major pathway through which genes affect external communities and ecosystem processes.

1.6 Rationale of the current study

1.6.1 The New Zealand context

Forests in New Zealand are composed of extensive native forest, which covers 6.8 million hectares (25% of land area) but also planted forest, that covers 1.7 million hectares (over 6% of land area) and an additional 5-10% of the land that includes native regenerating forests (FAO, 2015b). *Pinus radiata* is the most widely planted exotic forest species in New Zealand and it represents 88% of the exotic forest area, followed by Douglas-fir (*Pseudotsuga menziesii*) accounting for 6%, and the rest made up of cypress, eucalypts and other species (MPI (Ministry for Primary Industries), 2013). The predominance of *P. radiata* is explained by its high productivity (> 20 m^3 ha^{-1} yr^{-1}), great adaptability to soil and environmental conditions, responsiveness to silvicultural operations, strong gains per generation in the traits of interest to tree breeding, and good wood properties that lend themselves to a broad range of end-uses (Burdon *et al.*, 2008). Forest products, predominantly *P. radiata*, contribute approximately 3% to New Zealand GDP ($5 billion) and wood products are the third-largest export earner (behind dairy and meat products) (Forest Owners Association, 2016). Given the importance of the forestry industry in the country, over the last 60 years *P. radiata* has been subjected to intensive modern breeding, mainly by clonal propagation (Burdon *et al.*, 2008).

New Zealand exhibits a spatially and temporally heterogeneous climate and soil, all within a relatively small land area of 268,000 km^2. Annual and multi-year climate cycles present an historically record of significant variation. The Interdecadal Pacific Oscillation deviates by about 20%, with mean annual rainfall ranging between 600 and 6700 mm (1971-2000, National Institute of Water and Atmospheric Research) depending on the geographic location. Young soils are derived from recent volcanic, alluvial, glacial and coastal origins (Swift, 1991). In addition, New Zealand soil diversity is commonly characterised by deficiencies in mineral nutrients. The main nutrient deficiencies comprise nitrogen, phosphorus, magnesium and boron (Will, 1985), primarily in coastal sands, dredge tailings, where soils contain no or little organic matter. This variability in environment presents challenges for the effective management of the plantation forestry estate. The use of fertilisers is widespread to meet plant requirements, especially in the initial stages after tree establishment. Conventional plantation forestry management in New Zealand *recommends* N-fertilisers to be applied early in the rotation of *P. radiata* plantations, in concentrations around 70-184 kg N ha^{-1}, mostly in form of urea (200-400 kg urea ha^{-1}) and diammonium phosphate (400-500 kg DAP ha^{-1}), normally applied between spring and early summer (Maclaren, 1993).

In addition to the inherently variable climate in NZ, the prospect of climate change forecasts a strong likelihood of changes in current environmental patterns, mostly driven by the unpredictability of climatic events. Despite the fact that long-lived organisms, such as trees, have faced large inter-decade variation in climate and soil N dynamics during their evolution, modifications to already inherently variable environmental patterns via climate change may provide a significant challenge to tree establishment, survival and growth and hence forest ecology and production in the coming century. Understanding how trees and other long-lived woody plants locally respond to such change during their lifetime is a major focus for plant biology and ecology (Valladares *et al.*, 2007).

1.6.2 The need to understand G x E impacts on tree growth

In the coming decades, the increase in unpredictable environmental changes suggests that the improvement of sustainability will be an important challenge facing forestry. The application of chemical fertilisers and high-fossil fuel requirements have resulted in a range of

environmental costs. One of the most important global environmental concerns in recent decades has been the effect of the anthropogenic N inputs, associated with the constant increase in food demand, with consequences such as N deposition to the environment. Increased loads of N in ecosystem budgets through intensive N-fertilisation from agricultural systems are a threat to the quality of air, water and soil. N leaching into drainage water and the release of nitrous oxide and reactive N gases into the troposphere (NO_x, NH_3, N_2O) is accelerating the eutrophication of water, the acidification of soils (Robertson & Vitousek, 2009) and has global impacts on the climate system and on biodiversity in both land and aquatic ecosystems (Galloway *et al.*, 2008).

Another alarming impact of human activities and climate change is the threat to soil health. In 2015, declared as the International Year of Soils, the Food and Agriculture Organization for the United Nations (FAO) carried out the first major global assessment of soils (FAO, 2015c). In this, the FAO have reported that world's soils are rapidly deteriorating due to erosion, nutrient depletion, loss of soil organic carbon, soil sealing and other threats. Given the fact soils sustain 95 percent of food production, soil degradation is quantified as the most critical environmental threat to humankind. The connection of hydrological, biogeochemical and biogeophysical cycles indeed occurs to a large extent in soils, and is mediated by a huge diversity of microorganisms. Deteriorating soils has far-reaching consequences at many levels.

The environmental and economic input costs derived from nitrogen fertilisation increases the needs for improving the efficiency of agricultural and forestry production, even more in the future considering these costs will be greater as the resources potentially become scarcer. We need to develop an economically and environmentally sound use of valuable resources, such as water and nitrogen. These require a better understanding of the plasticity in traits related to nutrient use as well as genotype-specific traits that show better strategies of use in the face of variable environmental conditions.

The main goal of this thesis is to provide knowledge of how subtle tree genetic-based traits influence plant response to applied N sources with different chemical forms, and whether the resulting tree phenotypes extend to the root-associated microbial communities in the rhizosphere. The study of phenotypic plasticity in trees under changing soil nitrogen dynamics has important implications in terms of expanding the evolutionary un-

derstanding of how trees adapt to the environment based on the availability of N sources, but also may provide significant opportunities for enhanced tree establishment, survival and growth with positive impacts in soil ecology and long-term productivity robustness. Furthermore, the study of tree utilisation of a broader range of plant-available N pools across forest soils is important in the context of changing climate to improve sustainability, by the breeding selection of those genotypes/species with more efficient strategies of N nutrition.

1.6.3 Focussing questions

In an attempt to expand knowledge on the linkage between aboveground and belowground processes in tree nutrition, the present project was focused on three fundamental mechanisms involved in N nutrition. The following questions were addressed in experiments under both greenhouse and field conditions (except point 2):

1. *N utilisation.* Is the genotype-based variation in growth traits and resource distribution differently affected by differences in the form of N supply and associated environmental factors?

2. *N acquisition.* Can variations in N forms differently influence genetic root biotic and abiotic traits, such as the capacity to take up different N sources, root anatomical traits and the capacity to establish interactions with ectomycorrhizal fungi?

3. *N availability.* Does the impact of tree genetic variation, N environment and the interaction of both differently influence the associated rhizosphere microbiome?

1.7 Overview and thesis outline

In order to achieve these aims, two experiments were performed. The first one was a highly controlled glasshouse study over 6-months (Chapter 2 and Chapter 3) and the second one was a field trial established and managed over three years (Chapter 4 and Chapter 5). Both experiments were set up at the same time using 1-year old cuttings of ten commercially-available *Pinus radiata* genotypes. By using a similar experimental design, the purpose of the two experiments was to mirror and compare the variation in genotype-based growth

responses to N fertilisation with different N source: L-arginine - as the organic N form -, NH_4NO_3 - as the inorganic N form - and L-arginine:NO_3^- - as the N equimolar combination of organic and inorganic N forms. The study in Chapter 2 investigated whether variation in the N form applied influenced the capacity of N acquisition and the root biotic and abiotic traits across genotypes that showed contrasting growth responses to N source. The main aim in Chapter 3 was to characterise the rhizosphere microbiome of two *P. radiata* genotypes with distinct growth responses to N-fertilisation with different N forms, and determine whether the influence of N form, tree genotype and their interactive effects on growth extended to the richness, structure and composition of the root microbiome. The purpose of Chapter 4 was to sought evidence of variation in aboveground growth across the 10 genotypes after 2.5 years of fertilisation with different N source, and also to understand how incremental growth was altered by the field-scale variation in soil properties and resources distribution, in addition to seasonal weather fluctuations. The purpose of the investigations described in Chapter 5 was to better understand how bacterial and fungal communities in rhizosphere of two full-sib *P. radiata* genotypes, the same that were investigated in Chapter 3, were altered after 2-year fertilisation with different N source in a silt loamy soil, typically used for extensive farming and *P. radiata* plantations in New Zealand. Finally, in Chapter 6 I provide a synthesis of the findings and an explicit quantitative comparison of plant and microbiome responses in the greenhouse and field experiments.

Chapter 4 and Chapter 5 have been submitted for publications and are currently under review. Chapter 3 has been accepted for publication (subject to minor changes) in *Microbial Ecology*.

Chapter 2

Root characteristics influence genotype variation in response to nitrogen source in *P. radiata* (D.Don)

2.1 Introduction

Genetic differences in form and function within tree species have significant ecological implications (Whitham *et al.*, 2012). Among genotypes, phenotypic plasticity in response to changing environmental conditions influences tree growth (e.g. Hughes *et al.*, 2008; Bukowski & Petermann, 2014) resulting in strong changes in forest productivity (Fischer *et al.*, 2017). Furthermore, community studies have also indicated that plant genotypes exhibit specificity in the association with microbial communities, which suggests that host genotype is a major determinant regulating community, ecology and ecosystem functioning (e.g. Schweitzer *et al.*, 2008a, Chapter 3 and 5). However, fundamental questions, such as the significance of intraspecific variation in resource use and whether this implies niche differentiation among genetic individuals still remains unclear. The study of developmental plasticity to the availability of resources across genotypes is essential to predict species competition as well as to define genotype-specific traits that may improve adaptation to future environments.

Nitrogen (N) often limits net primary production in natural and managed terrestrial ecosystems in temperate and boreal zones (Vitousek & Howarth, 1991; Vitousek *et al.*, 1997). Soil is a heterogeneous, dynamic system and the diversity and availability of N forms is tightly linked to productive and consumptive processes (Moe, 2013); ranging from the simple inorganic forms (ammonium (NH_4^+) and nitrate (NO_3^-)) to more complex organic forms such as amino acids, peptides and proteins (Lonhienne *et al.*, 2015). Although it is well known that plants can use a wide range of N chemical forms, the abundance of

certain plant species in a particular habitat may be linked to the ability of plants to use the most available organic or inorganic nitrogenous compounds (McKane *et al.*, 2002).

Preference for the uptake of a particular N form has been defined by either an increase in uptake or superior growth when that N form is supplied (Britto & Kronzucker, 2013). For example, in boreal forests, where available plant N is predominantly in the form of amino acids (Inselsbacher & Näsholm, 2012), plants specialised in the uptake of organic N sources and absorb these compounds in considerable quantities (Näsholm *et al.*, 1998). In temperate forests, conifers such as *Pinus radiata* D. Don, perform more efficiently with ammonium than nitrate, based on greater uptake of ammonium and the poor competition with soil microbes for nitrate (McFee & Stone, 1968; Lavoie *et al.*, 1992). The reduced capacity of conifers to use nitrate has important consequences for reforestation in disturbed sites, in which the rise of soil pH increases soil nitrogen conversion from NH_4^+ to NO_3^- (Kronzucker *et al.*, 1997). Given the fact that organic N stocks are more variable and mineralisation is less constrained by low temperatures than in colder ecosystems, the plants capacity to take up organic N in temperate forests has captured less attention (Kielland, 1994; Näsholm *et al.*, 1998; Weintraub & Schimel, 2005). Furthermore, recent research in temperate tree species has demonstrated the capacity to take up amino acids at rates that often equal those of ammonium and nitrate (Scott & Rothstein, 2011). Although the relative contribution of organic and inorganic N sources in plant growth has not been directly addressed, the relative proportion of organic and inorganic N sources in soil (Finzi & Berthrong, 2005) as well as the species adaptation to changes in N fertility (Scott & Rothstein, 2011) are key factors that might determine preference for N forms.

Plant root systems have developed as highly complex networks that effectively explore and exploit soil resources as well as serving as a soil anchor. Roots have the ability to sense and respond to the soil heterogeneity by converting external stimuli to developmental responses through the plasticity of individual roots, rapidly adjusting the structure and function of the root system (Pregitzer *et al.*, 2002). Accumulation or depletion of soil nutrients (e.g. nitrogen) are detected by roots and in response, plants repress or promote root growth and lateral root development in order to enhance absorptive capacity (Drew *et al.*, 1973; McCormack & Guo, 2014). Plant N nutrition is further influenced by biotic factors tightly related to the associated plant belowground system, such as establishment

of symbiotic relationships with mycorrhizal fungi and competition with soil free-living microorganisms. The capacity of ectomycorrhizal (ECM) fungi to metabolise organic N compounds, including amino acids, enhances the plant access to N substrates (Sokolovski *et al.*, 2002; Talbot & Treseder, 2010). Although the role of mycorrhizal fungi in modulating root responses remains largely unknown, mycorrhizal associations have been reported to alter root morphology and extend fine root lifespan (Langley *et al.*, 2006). However, this is likely restricted to particular fungal species or types. Therefore, the effects of mycorrhizal associations on the adjustment of plant resources to root growth (Fogel, 1983) can have far-reaching effects on tree aboveground productivity.

There is a need to understand the physiological mechanisms that plants exhibit in environments with different nutrient compositions, as well as the linkages of these responses with growth. This requires an understanding of the genetic variation within individual species in terms of nitrogen use efficiency (NUE) and preference for different N sources. These have important implications for sustainability in agricultural and forestry management practices for two reasons: to contribute to the knowledge of genetic traits related to plant nutrition, and to assist in the selection of genotypes that show acclimation in terms of nutrient limitation across various environments.

In this study, I determined the intraspecific variation in the growth of ten *P. radiata* genotypes in response to additions of inorganic N, organic N and the equimolar combination of both N forms. Two hundred days after fertilisation, three genotypes with divergent growth responses to N form were selected and tested three different hypothesis to explain the observed genotype-specific tree responses. First, I addressed the question of whether N uptake and its internal distribution (determined using ^{15}N-labelled N compounds) varied across genotypes according to the growth response to N form. Second, I quantified if the level of ECM colonisation of roots differed across genotypes and N form supply. Third, I investigated whether the effects of the interaction between genotype and N form (G x N) on plant growth influenced root plasticity, by studying changes in the morphology of the root-cross sectional area.

2.2 Materials and Methods

2.2.1 Plant material and growing conditions

First, an experiment was conducted to investigate genotype variation in *P. radiata* growth in response to fertilisation with organic and inorganic N forms. Ten *P. radiata* genotypes were selected from those commercially available in New Zealand (provided by Forest Genetics Limited, New Zealand) previously selected in progeny trials based on growth rates, wood properties and disease resistance. The average biomass of the tree ramets before planting was 7.02 ± 0.83 g DW (4.43 ± 0.45 g shoot DW, 2.59 ± 0.42 g roots DW). In September 2013, trees were planted individually in 4 litre pots containing standardised unfertilised potting mix (15% bark, 50% pine fines, 15% cocoa fibre, 20% pumice) with good drainage and aeration to encourage root growth. Pots were grown in a fibreglass greenhouse located at the University of Canterbury (43°31'S, 172°35'E) under natural light, without temperature control (averaging 20°C in Summer and 18°C in Autumn) and with an automatic watering system that kept the growing medium continuously moist. From day 1 to 200 (November 2013 - April 2014), 12 replicate plants of each of the ten genotypes (360 individuals) were fertilised fortnightly with three different N treatments with equal N molarity (0.1 M N) estimated based on the plant N content (2 g N g^{-1} dry weight 100) required for growth and assuming 80% uptake (Table 2.1). Nitrogen treatments were (a) inorganic N (NH_4NO_3, 0.050 M), (b) organic N (L-arginine, 0.025 M) and (c) the factorial combination of equimolar N concentrations of organic and inorganic N forms (L-arginine:NO_3^-, 0.013:0.05 (M/M). N-fertilisation was undertaken by supplying 50-ml aliquots of each N solution with N-free Ingestad solution pH 5.5 (Ingestad, 1979). Nutrients were provided at the following concentrations: 1.35 g l^{-1} N, 0.17 g l^{-1} P, 0.87 g l^{-1} K, 3.37 mg l^{-1} Ca, 5.54 mg l^{-1} Mg, 3.78 mg l^{-1} S, 169.32 mg l^{-1} Fe, 6.08 mg l^{-1} Zn, 6.35 mg l^{-1} Cu, 98.37 mg l^{-1} Mn, 0.98 mg l^{-1} Mo, 250 mg l^{-1} B, 11.48 mg l^{-1} Cl and 1.75 mg l^{-1} Na. During the experiment pots were regularly moved around the greenhouse, and tree height and root collar diameter were measured monthly. After six months of fertilisation with different N forms, all replicates from three genotypes were selected for downstream analysis based on the most distinct growth rates in response to the N forms supplied. The selected genotypes showed (i) a positive response to organic N

and the combined treatment (genotype 15), (ii) a positive response to organic N but not to the combined treatment (genotype 31) and (iii) a neutral response to N form (genotype 48). None of the genotypes showed significantly greater growth response to inorganic N. Although eight out of ten genotypes had no significant effects of N form (genotypes 24, 28, 30, 35, 37, 44, 48 and 50), the criterion behind the selection of the genotype with neutral response was that genotype 48 shares full ancestry with genotype 31 (full-sib).

Table 2.1: Composition of nutrient solutions used for N-fertilisation and corresponding ^{15}N N-labelled sources applied in the 24h-uptake experiment. N forms within parenthesis show the presence of unlabelled N forms, at N equimolar concentrations.

Treatments	**N form**	**Label (1%)**
NH_4NO_3	100% NH_4NO_3	^{15}N-NH_4^+, ^{15}N-NO_3^-
L-arginine:NO_3^-	50% NO_3^-, 50% L-arginine	^{15}N-NO_3^- (L-arginine)
		U-[$^{13}C_6$], [$^{15}N_4$]-arginine (NO_3^-)
L-arginine	100% L-arginine	U-[$^{13}C_6$], [$^{15}N_4$]-arginine

2.2.2 Nitrogen uptake experiment

After day 215, an uptake study was undertaken. This consisted on two steps: a pretreatment with the N sources followed by the supply of the corresponding isotopically labelled N compounds. The pretreatment was undertaken for 36 h and consisted of the same N treatments used during plant growth of 0.1 M N concentration. Following pretreatment, an uptake experiment was conducted in nine out of the 12 replicates of each genotype (54 individuals) for 24 h, after adding ^{15}N and ^{13}C-labelled N sources with 1 % of isotopic enrichment (10 mM) that matched the N sources supplied during growth (Table 2.1): (a) NH_4NO_3 ($^{15}NH_4^+$ $^{15}NO_3^-$, > 0.98% ^{15}N) and (b) L-arginine (U-[$^{13}C_6$], [$^{15}N_4$]-L-arginine, > 0.99% ^{15}N). In order to evaluate the relative contribution of each N form in the combined treatment, four to five replicates (24 individuals) were treated with (c) L-arginine in the presence of unlabelled NO_3^- (U-[$^{13}C_6$], [$^{15}N_4$]-L-arginine, > 0.99% ^{15}N) and (d) NO_3^- in the presence of unlabelled L-arginine ($^{15}NO_3^-$, > 0.98% ^{15}N) (Persson *et al.*, 2006). The three remaining replicates were used to calculate ^{15}N and ^{13}C natural abundances.

2.2.3 Sample preparation and analyses

Trees were harvested 24 h after isotope addition and plant material was carefully divided in foliage, stem and roots. Foliage was subdivided in upper (Foliage^{u}) and lower needles (Foliage^{l}), taken from the middle point of the stem. Root systems were washed in running tap water and drained before measuring fresh weight (FW) (± 0.01 g). Half of the root system was stored in re-sealable plastic bags and kept moist before processing on the following days to assess the ECM colonisation rates and study root anatomical descriptors. Dry weight partitioning of biomass fractions (needle, stem and root) were obtained after oven-drying the plant tissues at 60°C for 48 h (± 0.01 g). Subsamples of needle fractions and fine roots (300 mg) were used for the analysis of N and C content using an elemental analyser (Elementar Isoprime 100 analyser, Isoprime, UK) after being homogenised, freeze-dried and pulverised with a ball mill to a fine powder. Sample δ^{15}N and δ^{13}C values were obtained after combustion of the samples, separation by gas chromatography and analysis by continuous-flow mass spectrometry (Europa Scientific 20/20 isotope analyser, Europe Scientific, Crewe, UK) at the University of Waikato (Hamilton, New Zealand). The internal distribution of ^{15}N/^{13}C was calculated by subtracting the natural abundances of the heavier isotopes from the atom % in each labelled sample. N and C acquisitions were calculated as the total measured plant ^{15}N/^{13}C relative to root dry weight (DW).

2.2.4 Ectomycorrhizal root colonisation and measures in root-cross sections

Roots from six of the 12 individual genotype replicates grown in each of the N treatments were randomly selected to perform the root analyses and kept in deionized water to prevent desiccation (108 samples). Individual roots were dissected and classified by order following the convention (Pregitzer *et al.*, 2002) that numbers distal roots as first-order roots, increasing sequentially with each branch from distal to proximal portions of the root system. Only the first three orders of roots were studied because of the high variation in root size of fourth-order roots. Roots from each of the individual trees were isolated by root order, pooled depending on the genotype and the N form and stored in re-sealable plastic bags with wet tissue in order to keep the moisture.

Approximately 1 g of roots sorted by order were placed in tubes containing 10% (v/v) ethanol solution at 4°C and used to assess the ectomycorrhizal (ECM) root colonisation at a later date. The quantification of ECM root colonisation was done by the gridline intersection method (Brundrett *et al.*, 1996). Fine roots were cut into 1-cm pieces, arranged lengthwise in a thin layer at the gridline and examined under a dissecting microscope with transmitted light. Approximately 300 intersections were assessed for each root order, genotype and N form. The proportion of root lengths colonised by ECM fungi was calculated as the percentage of intersects with mycorrhizal tips over the total number of intersects.

Approximately 2 g of roots were used to study the cross-sectional anatomy of roots. The analysis comprised roots from genotypes 15, 31 and 48 genotypes grown in L-arginine and NH_4NO_3 (93 samples), but not those grown in the combined treatment, due to the long processing time required for the dissection. Cross-sections from the three root orders were free-hand dissected using a thin shaving razor-blade while subjecting roots between two carrot pieces, in order to keep the shape and the moisture of root cells. Root cross-sections were kept in water before the selection under the dissection microscope of clear cuts, thereby those with one-cell layer and no signs of cell deterioration. Root sections were cleared in 10% KOH (v/v) at 80°C for 60 to 90 minutes on a heating plate under a fume cupboard. After cooling down, roots were rinsed in tap water and kept in 70% glycerol before the image capture. In order to better distinguish the stele and cortex areas, cross-sections were placed on microscope slides, stained in Toluidine blue 0.1% (in 70% glycerol) and rinsed with deionised water, before a cover slip was added prior to examination under a Leica M125 stereo microscope (Leica Microsystems CMS GmbH, Germany). Pictures were taken using the coupled Magnifier in DV camera Leica DFC and Leica Application Suite V4.3. Image analysis of roots cross-sections was performed using ImageJ 1.48 (Schneider *et al.*, 2012) by adjusting the image scale and setting color threshold to measure the contrast between root areas. Cortex area was obtained by subtracting the stele area from the root area. Images in Figure 2.6 were processed in the same way but were observed using a Zeiss AxioImager.M1 compound microscope (Carl Zeiss MicroImaging, Göttingen, Germany). Images were acquired using a Zeiss AxioCam HRc CCD camera and AxioVision Rel. 4.5 software.

2.2.5 Statistical analysis

The statistical analysis was performed in R Studio (Version 0.99.485). To test whether *P. radiata* genotypes differed in response to the effect of N forms (tree height, diameter, plant biomass and associated descriptors, N and C contents, ^{13}C and ^{15}N and root colonisation), linear models were used after confirmation that data met assumptions for normality and homogeneity of variance. Linear models included genotype, N supply and the interaction of genotype and N form as explanatory variables. When the interaction was significant the analysis focused on how responses varied between genotypes using *lsmeans* package (Lenth, 2016) and *posthoc* Tukey pairwise contrasts. Principal components analysis (PCA) ordination was used to visualise relationships in the plant distribution of biomass dry weight across N treatments and genotypes. The ordination was based on Euclidean distance and was performed with the prcomp function (stats package) applied to scaled and centered (with zero mean and unit variance) biomass measures. A two-way multivariate analysis of variance (MANOVA) was conducted to test whether biomass across tissues differed between N treatments or tree genotypes. Linear regression models were used to examine the effects of N treatment in the ECM colonisation of roots (in each of the root order), while the relationship between cortex and stele areas was only studied comparing between the sole organic and inorganic N forms.

2.3 Results

2.3.1 Growth in response to different nitrogen chemical forms

After six months of growth in the different N form environments, trees showed an averaged (mean ± SEM) height growth of 62.22 ± 0.48 cm and root collar diameter of 8.74 ± 0.54 mm (Table 2.2). Height growth response was influenced by a significant effect of the interaction between genotype and N form (G x N)($F_{(18,330)} = 2.12$, $p < 0.001$). Conversely, diameter growth was influenced by tree genotype ($F_{(9,330)} = 3.69$ $p < 0.001$), but not N form ($F_{(2,330)} = 2.32$, $p = 0.415$). The height growth response in individual genotypes revealed that N form increased height growth in genotypes 15 ($p < 0.001$) and 31 ($p = 0.50$), with an enhanced growth under organic N compared to NH_4NO_3 supply. Height

growth response to the combination of organic and inorganic N forms (L-arg:NO_3^-) in genotype 15 was similar to that in the sole L-arginine treatment ($p < 0.001$), but had no effect on height growth in genotype 31 ($p = 0.925$). In order to investigate the effect of N form on biomass partitioning, three genotypes were harvested based on the height growth response to N form. Genotypes selected were (i) genotype 15, with greater height growth in response to L-arginine and L-arginine:NO_3^- compared to the inorganic N form and (ii) genotype 31, with greater height growth in response to L-arginine compared to that under NH_4NO_3, but no effect of the combined N treatment. Because of the lack of genotypes with a greater response to NH_4NO_3, the third genotype selected was (iii) genotype 48, a full-sib of genotype 31 that showed similar height growth response to organic and inorganic N forms.

Table 2.2: Height and root collar diameter growth across the ten *P. radiata* genotypes in response to N form. Mean values ± SEM are shown. Means within a row followed by different letters indicate significant differences in response to N treatment ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

	Height (cm)			Diameter (mm)		
Genotype	NH_4NO_3	L-arg:NO_3^-	L-arginine	NH_4NO_3	L-arg:NO_3^-	L-arginine
15	55.5±1.9**b**	65.3±3.2**a**	67.1±2.3**a**	8.61±0.22	9.72±0.37	9.37±0.19
24	64.8±3.0	61.7±2.1	69.3±2.9	9.21±0.21	9.30±0.19	9.05±0.18
28	77.2±1.4	77.3±2.1	75.0±2.4	10.22±0.19	10.07±0.24	10.0±0.28
30	60.4±1.9	58.3±1.7	60.9±2.3	9.57±0.35	9.29±0.27	9.6±0.28
31	60.4±2.4**b**	61.6±1.3**ab**	67.7±1.5**a**	8.39±0.23	8.92±0.28	9.00±0.15
35	71.9±3.4	69.5±2.4	67.3±1.2	8.95±0.22	8.89±0.20	8.8±0.23
37	61.8±2.8	60.2±3.3	65.7±2.4	8.05±0.18	8.43±0.26	8.9±0.26
44	61.6±1.6	59.9±1.6	61.5±1.8	8.89±0.36	8.34±0.28	8.42±0.23
48	64.1±2.6	64.1±2.6	66.2±1.4	8.86±0.30	8.68±0.19	8.73±0.20
50	68.9±2.8	65.6±1.3	69.1±2.5	9.3±0.20	9.34±0.23	9.6±0.20

Similar to the differences found in height growth, plant total biomass was influenced by the interactive effect between genotype and N form ($F_{(18,330)} = 2.56$, $p = 0.043$) (Figure 2.1). Genotypes 15 ($p = 0.013$) and 31 ($p = 0.062$) showed greater biomass when supplied with L-arginine compared to NH_4NO_3, while genotype 48 did not respond to N-fertilisation (Figure 2.1). Because of the non-significant effect of N form in root dry weight (N supply - $F_{(2,330)} = 0.41$ $p = 0.667$, G x N - $F_{(18,330)} = 1.36$, $p = 0.252$) the difference in total biomass was due to intraspecific variation in aboveground biomass allocation. Shoot biomass was significantly influenced by the interaction between genotype and N form (Shoot biomass - $F = 3.51$ $p = 0.009$). This interaction was driven by the

significantly greater stem dry weight in genotypes 15 ($p < 0.001$) and 31 ($p = 0.049$) under fertilisation with L-arginine compared to NH_4NO_3. The different aboveground allocation across the genotypes also influenced foliage biomass (G x N - $F_{(18,330)} = 2.45$, $p = 0.049$), in which genotypes 15 ($p = 0.002$) and 31 ($p = 0.077$) showed greater foliage dry weight in the L-arginine treatment than in the NH_4NO_3 treatment. In genotype 15, trees fertilised with the combined treatment also showed greater stem ($p < 0.001$) and foliage ($p = 0.034$) biomass in response to L-arginine:NO_3^- compared to NH_4NO_3 and L-arginine, respectively. In contrast, the effects of the combined treatment for genotype 31 did not influence the allocation in stem and foliage compared to the other N treatments. Finally, the main source of variation in root biomass was driven by genotype, in which genotype 48 showed the greatest root biomass followed by genotypes 31 and 15.

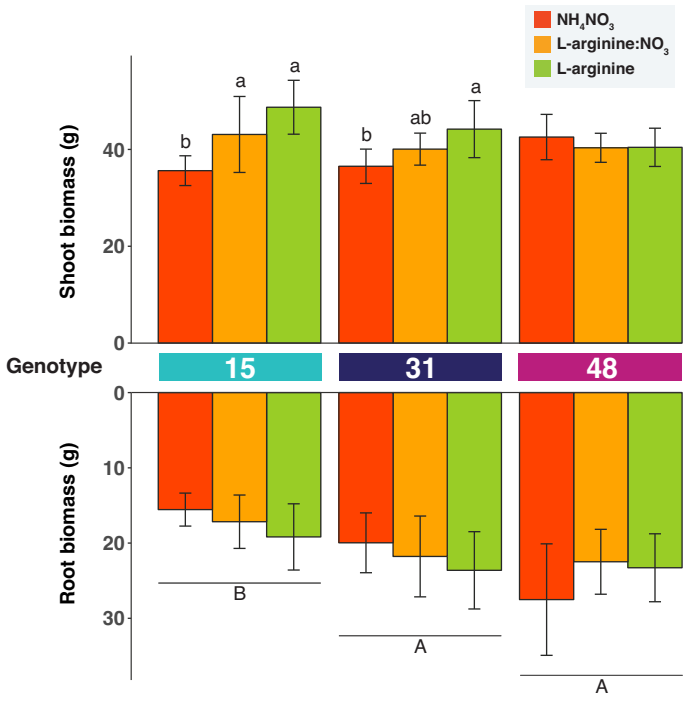

Figure 2.1: Shoot and root biomass of *Pinus radiata* genotypes, after 200 days of periodic N-fertilisation with different N forms. Bars represent average values ± SEM, N = 12 for shoot biomass and N = 10 for root biomass. Different lower-case and upper-case letters indicate significant differences in response to N treatment and genotype, respectively, ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

On average, 41.8% of total biomass was in the foliage fraction, 25.1% in the stem fraction, including main stem and lateral branches, and 33.1% in the root fraction. In

contrast to the significant differences found in absolute biomass dry weight, the study of biomass partitioning (Table 2.3) revealed that genotype was the main source of variation across foliage, stem and root fractions, with no significant differences displayed between N forms (Foliage - $F_{(2,330)} = 3.29$, $p = 0.041$; Stem - $F_{(2,330)} = 17.16$, $p < 0.001$; Root - $F_{(2,330)} = 17.16$, $p < 0.001$). Similarly, the root-to-shoot ratio differed by tree genotype ($F_{(9,330)} = 15.23$, $p < 0.001$), increasing from genotype 15 to 48.

Table 2.3: Total biomass, biomass fractions and N content across plant tissues and root:shoot ratio at harvest of *P. radiata* genotypes, 200 days after periodical N-fertilisation with different N forms. Mean values ± SEM are shown. Different lower-case and upper-case letters indicate significant differences in response to N treatment and genotype, respectively, ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

	Genotype 15			Genotype 31			Genotype 48		
	NH_4NO_3	L-arg:NO_3^-	L-arginine	NH_4NO_3	L-arg:NO_3^-	L-arginine	NH_4NO_3	L-arg:NO_3^-	L-arginine
Biomass	51.0±2.4^{b}	60.3±4.8^{a}	67.8±4.9^{a}	58.5±2.7^{b}	61.8±3.3^{ab}	71.8±4.2^{a}	70.1±5.0^{a}	62.8±3.2^{a}	64.6±3.8^{a}
Root frac	30.5±1.1^{B}	28.6±1.4^{B}	27.9±1.1^{B}	33.7±1.7^{A}	34.3±1.9^{A}	32.5±1.3^{A}	38.2±2.3^{A}	35.2±1.3^{A}	35.6±1.1^{A}
Root N	1.01±0.04	0.88±0.04	0.91±0.04	0.96±0.04	0.98±0.02	0.97±0.03	0.93±0.03	0.93±0.04	1.01±0.06
Foliage frac	43.0 ±1.7	41.4±1.3	43.4±1.2	42.9±1.5	43.5±1.6	42.0±0.8	38.4±1.7	42.3±1.5	39.8±1.4
Foliage^{u} N	1.20±0.07	1.11±0.05	1.06±0.07	1.06±0.05	1.12±0.02	1.12±0.05	1.14±0.05	1.12±0.04	1.24±0.05
Foliage^{l} N	1.11±0.06^{a}	0.93±0.03^{b}	1.02±0.07^{ab}	1.10±0.04^{a}	1.16±0.03^{a}	1.09±0.04^{a}	1.11±0.06^{a}	1.10±0.04^{a}	1.23±0.05^{a}
Stem frac	26.5±1.3^{A}	30.0±1.6^{A}	28.7±0.7^{A}	23.4±1.0^{B}	22.2±0.8^{B}	25.5±1.3^{B}	23.4±1.4^{B}	22.5±0.6^{B}	24.6±0.9^{B}
Root:shoot	0.44±0.02^{B}	0.41±0.03^{B}	0.39±0.02^{B}	0.52±0.04^{A}	0.54±0.06^{A}	0.49±0.03^{A}	0.64±0.06^{A}	0.55±0.03^{A}	0.56±0.03^{A}

A principal coordinate analysis (PCA) ordination was generated to visualise relationships among samples in terms of biomass allocation (Figure 2.2). While samples of plant tissues varied in their dry weight biomass, samples from trees treated with L-arginine tended to cluster separately from trees treated with NH_4NO_3 on PC1, but not with samples treated with L-arginine:NO_3^- (Figure 2.2a). Samples from genotype 15 tended to cluster separately from genotypes 31 and 48 on PC2 (Figure 2.2b). These patterns were confirmed by a MANOVA, which showed that biomass dry weight across plant tissues differed between N form ($p = 0.007$) and tree genotype ($p < 0.001$). However, there was no significant interaction between N treatment and tree genotype for distance between samples based on biomass dry weight ($p = 0.086$).

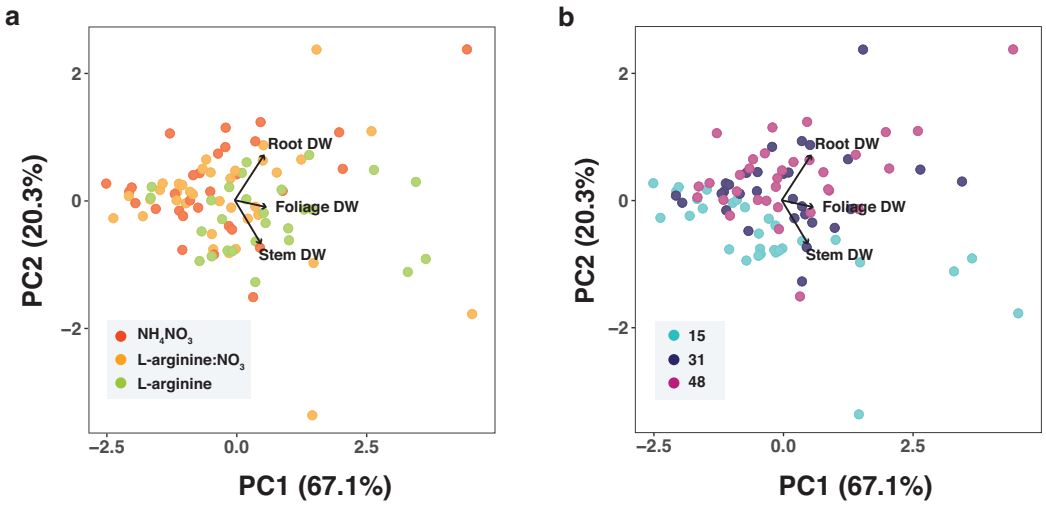

Figure 2.2: Principal component analysis ordination biplot of distances between samples displayed by N form (a) and tree genotype (b). The percentage of variation explained by each principal component is given in parentheses. Distributions of biomass dry weight across plant tissues are represented by vectors, and length and direction of vectors can be interpreted as correlations.

2.3.2 Capacity for nitrogen acquisition

The amount of recovered ^{15}N as well as the rate of N acquisition (by unit of root dry weight) 24 h after the addition of ^{15}N-labelled compounds, showed little variation in response to ^{15}N-labelled N source or tree genotype (Figure 2.3). Rates of N acquisition had an average (mean ± SEM) of 0.13 μmol ^{15}N g DW^{-1} ± 0.01 for the two sole N forms (L-arginine and NH_4NO_3), and 0.14 μmol ^{15}N g DW^{-1} ± 0.02 for the combined treatments (^{15}N-NO_3^-

in presence of L-arginine and U-[$^{13}C_6$], [$^{15}N_4$]-arginine in presence of NO_3^-). In addition, no statistical differences were found in the N content of roots and upper foliage across N treatments (Table 2.3).

The internal distribution of ^{15}N exhibited differences across plant tissues (Figure 2.3). Roots had the greatest content of ^{15}N (up to 15 μmol excess ^{15}N) followed by the upper and the lower needles. There was a slightly greater allocation to leaf tissue in the mid-to-upper portion of the stem (upper needles - up to 0.2 μmol ^{15}N excess; lower needles - 0.15 μmol ^{15}N excess). ^{15}N could not be detected in the needles of genotype 15 in any of the combined treatments (Table 2.3). The distribution of ^{15}N in upper needles differed across the combined treatments (Figure 2.3a), depending on whether the ^{15}N-labelled molecule was L-arginine or NO_3^- ($F_{(1,18)} = 9.57$, $p = 0.006$), and also the genotype ($F_{(2,18)} = 4.11$, $p = 0.037$). In the combined treatments, upper needles possessed a greater proportion of the ^{15}N labelled when the NO_3^- was taken up in presence of L-arginine than in the opposite situation, both in genotype 31 ($p = 0.008$) and 48 ($p = 0.012$). Finally, the distribution of ^{15}N in lower needles across the sole N treatments (Figure 2.3b) showed no significant interactive effects.

Differences in the ^{13}C distribution and the rate of ^{13}C acquisition were found in the equimolar N treatments that added double N-labelled L-arginine molecule^{13}C (N-labelled L-arginine and N-labelled L-arginine in presence of NO_3^-)(Table 2.4). The rate of ^{13}C acquisition (by unit of root dry weight) differed between genotypes ($F_{(2,18)} = 5.78$, $p = 0.009$), with genotype 15 displaying a greater rate of ^{13}C acquisition compared to genotype 31 ($p = 0.007$). In addition, the internal distribution of ^{13}C in needles showed a significant effect of the genotype by N form interaction ($F_{(2,18)} = 5.67$, $p = 0.015$) that led to a greater level of ^{13}C in lower needles of genotype 15 ($p = 0.003$), when this was supplied with NO_3^- in contrast to L-arginine alone.

2.3.3 Root plasticity in response to nitrogen form

2.3.3.1 Ectomycorrhizal root colonisation

The main sources of variation in the level of ECM root colonisation (see Figure 2.6d-f) were root order ($F_{(2,93)} = 63.29$, $p < 0.001$) and N form ($F_{(2,93)} = 22.91$, $p < 0.001$). Genotype had no influence in colonisation levels ($F_{(2,93)} = 0.71$, $p = 0.501$). A significant negative

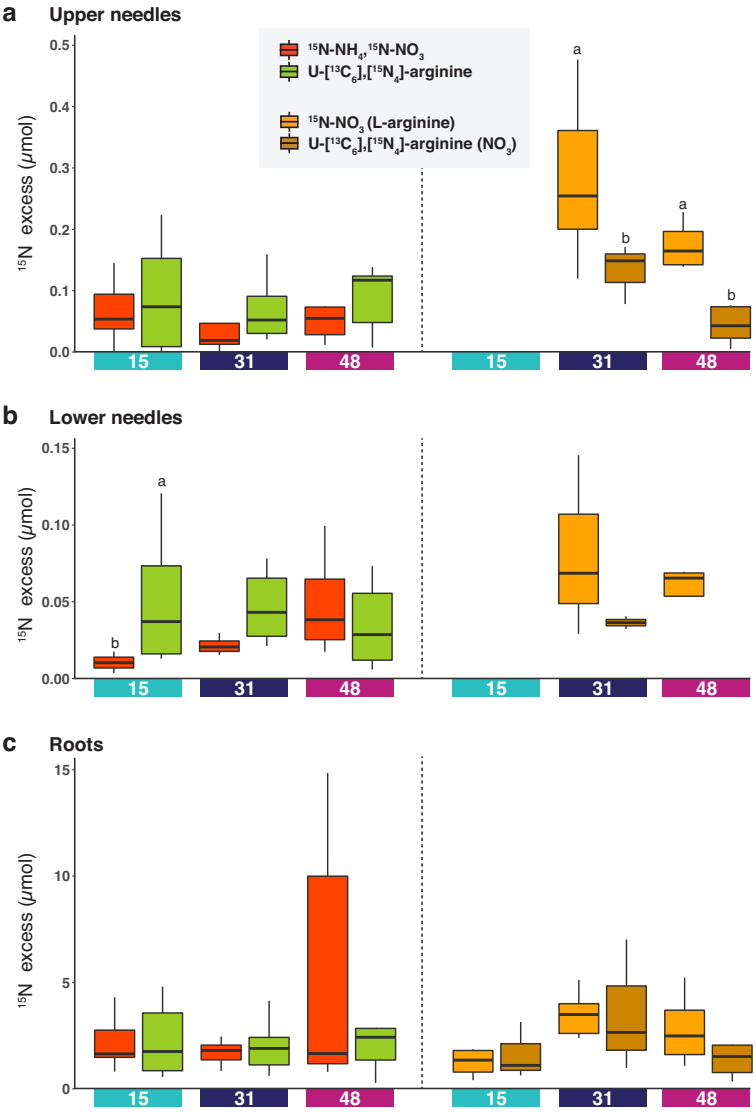

Figure 2.3: Internal distribution of ^{15}N across *P. radiata* genotypes, 24 hours after the application of ^{15}N N-labelled sources. Left-hand plots show the differences in distribution across sole equimolar N-labelled N sources, while right-hand plots show differences in ^{15}N distribution across the combined organic and inorganic N supplies. The bottom and top edges of the boxes mark the first and third quartiles, while the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above boxes indicate significant ($p < 0.05$) differences using 2-way ANOVA followed by Tukey *post hoc* pairwise comparison.

correlation (Figure 2.4, Figure 2.6) was found between the level of root colonisation and root order (Pearson's correlation - r = -0.78, $p < 0.001$). The form of N supply influenced the degree of colonisation across the studied root orders (First order, $F_{(2,30)} = 5.76$, $p = 0.024$; Second order, $F_{(2,30)} = 6.78$, $p = 0.016$; Third order, $F_{(2,30)} = 7.69$, $p = 0.004$). Roots from trees grown in L-arginine showed a consistently greater ECM colonisation

Table 2.4: Distribution of ^{13}C (μmol ^{13}C excess) and rate of ^{13}C uptake (μmol ^{13}C excess g^{-1} root DW) 24 hours after the application of $^{13}C^{15}N$ -double labelled L-arginine, either in absence (L-arginine) or presence (L-arg:NO_3^-) of nitrate. Mean values ± SEM are shown. Different lower-case and upper-case letters indicate significant differences in response to N treatment and genotype, respectively, ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

	Genotype 15		Genotype 31		Genotype 48	
	L-arginine	L-arg(NO_3^-)	L-arginine	L-arg(NO_3^-)	L-arginine	L-arg(NO_3^-)
Foliage^{u}^{13}C	-	-	3.301±1.228	-	-	3.038±3.037
Foliage^{l}^{13}C	2.854±1.067^{b}	7.873±0.268^{a}	-	-	-	3.506±1.161
Root^{13}C	4.284±2.190	5.782±0.522	-	-	6.009±2.406	7.407±1.877
Uptake^{13}C	0.600±0.179^{A}	0.865±0.062^{A}	0.155±0.051^{B}	-	0.305±0.131^{AB}	0.593±0.275^{AB}

(First order, $p = 0.021$; Second order, $p = 0.035$; Third order, $p = 0.011$) compared to roots from NH_4NO_3-treated trees, while the effect of the combined treatment increased the level of root colonisation in second and third-order roots compared to roots treated with NH_4NO_3 (First order, $p = 0.141$; Second order, $p = 0.021$; Third order, $p = 0.005$).

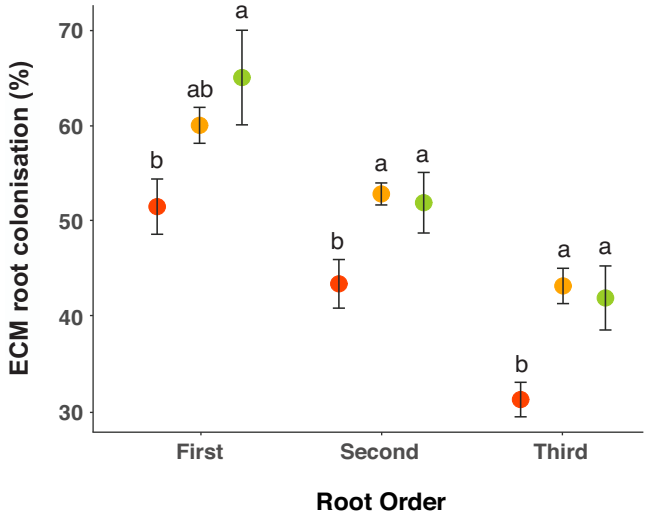

Figure 2.4: Effect of N form on the relationship between the degree of ECM root colonisation and root order. Fertilisation was carried out during 200 days with either NH_4NO_3 (red dots), L-arginine:NO_3^- (orange dots) and L-arginine (green dots). Mean values ± SEM are shown. Different lower-case letters indicate significant differences between N treatments in each of the root orders ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

2.3.3.2 Areas of the root-cross sections

The study of plasticity across root samples (see Figure 2.6a-c) revealed that root cross-sectional area was significantly influenced by the effects of genotype ($F_{(2,230)} = 67.73$, $p < 0.001$), N form ($F_{(1,230)} = 4.84$, $p = 0.028$) and root order ($F_{(2,230)} = 319.65$, $p <$

0.001) with no significant interactive effect between genotype and N form (F = 1.16, p = 0.313)(Table 2.5). The genotype effect in root area changed according to the measured root dry weight (see section 2.3.1, Figure 2.1), increasing from genotype 48, 31 and 15. The lack of significant interaction indicated that differences in root area across N forms were consistent among genotypes. Genotypes showed consistently greater root area in trees grown in L-arginine than those fertilised with NH_4NO_3, although the significance of such responses varied depending on the genotype and the root order (Table 2.5). In addition, the stele-to-cortex ratio of the root area differed between N forms. The positive relationship between the cortex and stele partitioning of root area (Figure 2.5) showed a distinct relationship depending on the N form of the N-fertiliser. Roots from trees treated with organic N had generally greater stele relative to cortex area compared with plant roots grown in inorganic N.

Table 2.5: Cross-sectional area across root orders of *P. radiata* genotypes in response to N treatment. Different lower-case letters across rows indicate significant differences between N treatments ($p < 0.05$) based on *post hoc* Tukey pairwise comparison.

	Genotype	**Root Area (mm^2)**	
		NH_4NO_3	L-arginine
Order 1	15	**0.21±0.01**^{b}	**0.26±0.02**^{a}
	31	0.21±0.01	0.21±0.02
	48	**0.32±0.01**^{b}	**0.46±0.02**^{a}
Order 2	15	**0.44±0.03**^{b}	**0.55±0.02**^{a}
	31	**0.33±0.01**^{b}	**0.55±0.02**^{a}
	48	**0.45±0.01**^{b}	**0.74±0.04**^{a}
Order 3	15	0.59±0.06	0.50±0.03
	31	1.06±0.07	1.54±0.12
	48	**1.79±0.07**^{b}	**2.07±0.20**^{a}

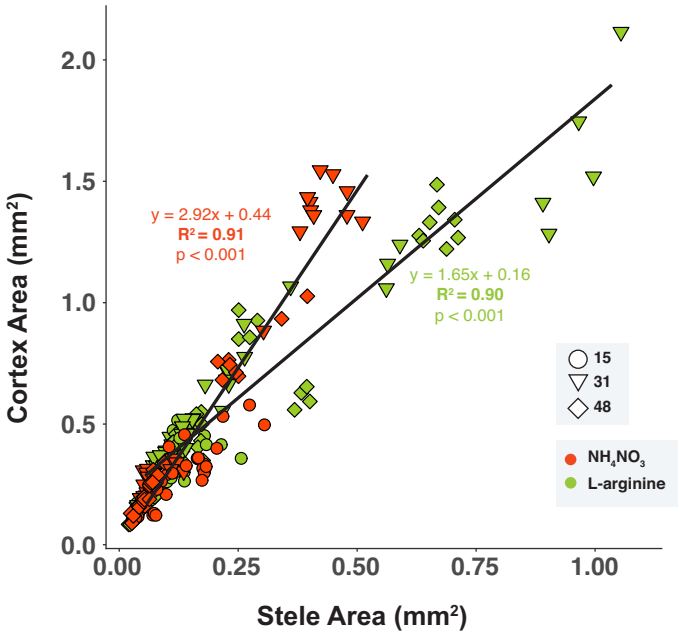

Figure 2.5: Shift on the relationship between cortex and stele area in response to N form. A total of 45 root-sections were measured in each genotype and for every N form. Regression equations, coefficients of determination (R^2) and p-values are displayed next to each linear regression for each N form.

2.4 Discussion

Over recent decades, many research efforts have focused on investigating species preference for N forms and the availability of organic N forms, especially amino acids, through many ecosystems (McKane *et al.*, 2002; Weigelt *et al.*, 2003). In contrast, very little research has examined the intraspecific variation in response to different N forms, despite the ample adaptive strategies that plant genotypes have been shown to exhibit to a wide range of environments (e.g. Abrams, 1994). Results from this study provide evidence of genotype variation in growth response (biomass dry weight) to the effects of equimolar concentrations of L-arginine and NH_4NO_3 and expand the knowledge of specific patterns of root plasticity in response to N form. From the ten genotypes screened in this experiment, two of them had greater biomass when grown in L-arginine compared to NH_4NO_3, while the others responded similarly to both N sources. In addition, genotypes with enhanced growth in response to L-arginine (genotypes 15 and 31) showed different responses to the effect of the combined treatment (L-arginine:NO_3^-), which indicates that the use of NO_3^- is also influenced by tree genetics. There is a significant lack of understanding on how

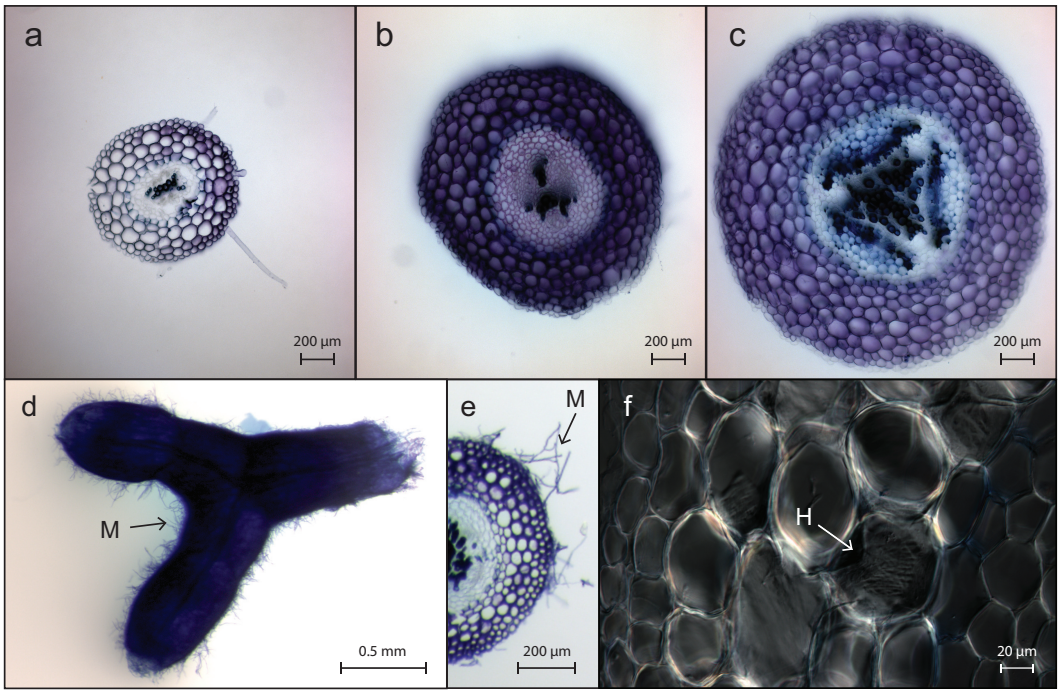

Figure 2.6: Micrographs of root sections of *P. radiata*. Images (a-e) were captured using bright field 10x objective, and (f) differential interference contrast (DIC) illumination 40x objective. First (a), second (b) and third (c) order-roots dissected and classified following the convention (Pregitzer *et al.*, 2002). (d) Root tip colonised with ectomycorrizal fungus with several layers of hyphal mantle (M). (d) Second order-root section covered by a thick fungal mantel (M). (f) Higher-magnification view of ECM root-section showing the labyrinth in Hartig net hyphae (H) around epidermal cells.

N forms influence biomass distribution in trees, and findings from studies of the effect of different inorganic N sources, alone or in combination, are not consistent (e.g. Bauer & Berntson, 2001; Bown *et al.*, 2010). Studies in conifers indicate trees grow better under ammonium than nitrate (McFee & Stone, 1968; Kronzucker *et al.*, 1997), although there are exceptions to this (Parfitt *et al.*, 2003). This suggests that despite the generally poor uptake, utilisation and storage capacity for NO_3^- in conifers, there is a degree of variation in plant responses to NO_3^- availability (Britto & Kronzucker, 2013).

A number of studies (e.g. Öhlund & Näsholm, 2001; Cambui *et al.*, 2011) have reported that young seedlings fertilised with organic N and NH_4^+ in combination with NO_3^- leads to similar biomass dry weight, although root partitioning and/or root-to-shoot ratio may be increased in response to organic N. In the present study, genotypes with enhanced growth under organic N supply showed greater allocation in shoots (stem and

foliage biomass), while changes in biomass partitioning were only influenced by the effect of tree genotype. The similar plant N content and root-to-shoot ratio across N forms in genotypes with positive response to organic N suggests L-arginine led to an overall greater tree size compared to NH_4NO_3. Likewise, genotypes with neutral response to N form did not show differences in biomass partitioning after growing under organic and inorganic N treatments. I believe that the reason behind the divergences with previous studies might be the different growing conditions and plant genetic material, considering this study was conducted in clonally-propagated trees and new root growth in these root systems could be unrepresentative of 1-year old seedlings. Differences in biomass allocation vary over the plant lifecycle but also in response to changes in the environment and among species (Reich *et al.*, 2003). Considering this, an ample range of confounding effects could be driving shifts in allocation patterns. For example, a very strong allocation response is induced when nutrients are limiting in the environment. As a consequence, plants increase allocation in roots at the expense of stem and leaf biomass. Although this could be a possible explanation for the observed different growth patterns in genotypes with a significant response to organic and inorganic N, the generally similar (and rather moderate) N content levels in new-growth needles across individuals suggest comparable N status between N treatments/genotypes. Nevertheless, it provides evidence that genotypes with greater aboveground biomass growth in response to L-arginine compared NH_4NO_3, were more efficiently using the added N as they received equimolar N concentrations of both N forms.

It would be interesting to speculate whether genotypes with and active growth response to one N form over the other are the result of the interaction between the plant genetic background and the specialisation to the environment. This would result in the impaired/restricted capacity to uptake/metabolise one N form over the other. This could reveal a range of interesting interactions between different plant traits related to the N use (i.e. N uptake, N translocation, N assimilation, N allocation, root C exudation, symbiosis with mycorrhizal fungi) and their suitability to respond to a particular N form. The significance of the apparent specialisation and the underlying mechanisms driving the interaction would need to reverse in response to the other N form, as transitive changes can be activated and inhibited. This could be mediated through genetic and epigenetic

changes (e.g. gene expression, changes in chromatin, changes in transposable elements).

The results here support previous evidence that conifers have a notable degree of flexibility in the uptake of different N forms (Boczulak *et al.*, 2014), although the capacity for N uptake by the three genotypes did not fully explain the observed intraspecific variation in growth. This is not completely surprising, because to-date improvements in N acquisition and NUE in crops using metabolic engineering of nutrient transporters have not succeeded in increasing yield (Britto & Kronzucker, 2004), demonstrating that a superior N acquisition is not directly related with an increase in growth. Furthermore, regardless of the ^{15}N uptake, the distribution of ^{15}N in needles differed across the combined treatments, indicating a preference for shoot allocation of ^{15}N-NO_3^- (in presence of L-arginine) in genotypes 31 and 48, compared to N-labelled L-arginine (in presence of NO_3^-). These results are supported by studies (Cambui *et al.*, 2011) that indicate NO_3^- has a different site for primary assimilation than L-arginine, so that NO_3^- may be directly transported to the shoot, while amino acids may be preferentially metabolised in roots.

The findings here make it difficult to assess the extent to which *P. radiata* genotypes take up L-arginine as an intact molecule, as observed by the non-significant regression between ^{13}C and ^{15}N excess suggested by Näsholm *et al.* (1998). In this, the assumption of a theoretical slope of 1.5 corresponds to 100% N uptake as L-arginine (C_6:N_4) while deviations of slope estimate the fraction of nitrogen taken up as intact amino acid. Given that the uptake experiment was performed by directly adding labelled sources into the potting mix and not into sterile conditions, either soil biotic or plant metabolic factors could influence the availability/uptake of intact N sources. Factors such as root transpiration and NH_4^+-efflux after the absorption of amino acids could influence the observed non-significant ratio between ^{13}C and ^{15}N excess. However, it is worth mentioning that the present experiment used a 24-h time period between the addition of labelled compounds and the harvesting of trees and this might not be appropriate to evaluate the level in which L-arginine was taken up as an intact molecule. This is because amino acids are very dynamic in soil and their turnover is estimated to occur within the 4-6 h after the addition of labelled N-compounds (Kielland *et al.*, 2007). In addition, rhizosphere microbes can easily compete with plants for amino acids (Harrison *et al.*, 2008) and also assist in the conversion between N forms, such as by the mineralisation of organic N sources before

the uptake took place. However, given the wide functional diversity of microorganisms in the rhizosphere as well as the different soil N processes that mediate, the biotic and abiotic factors that might differently influence the availability and uptake of organic and inorganic N source are still the subject of much-needed study, as is investigated further here in Chapter 3 and 5.

Other biotic factors, such as the symbiotic relationship between plants and mycorrhizal fungi, can also improve the uptake capacities of fine roots by depolimerising and making more accessible complex N structures (Read & Perez-Moreno, 2003; Smith & Read, 2008). This mechanism may provide an explanation for the results in this study, in which the effect of N form influenced the extent of ECM root colonisation. Consistently greater colonisation rates were found in response to organic N compared to the inorganic N treatment, as well as in response to the combination of NO_3^- and L-arginine compared to NH_4^+ and NO_3^-. The high rates of ECM root colonisation have been previously observed in response to amino acids supply (Turnbull *et al.*, 1995; Dickie *et al.*, 1998). This suggests that a potential restriction/competition on a plants capacity to use amino acids as N sources might be enhanced because of the symbiotic association with ECM. However, given the lack of N-free control, it is unclear whether this is a result of the repression of colonisation by NH_4^+ or the induction by L-arginine. It is important to notice that despite mycorrhizal associations being generally considered mutualistic relationships, the N:C exchange ratio varies depending on the soil N availability. For instance, in N-limited conditions the fraction of N provided to the host plant by mycorrhizae has been reported to decline, contributing to fungal N-immobilisation rather than mobilisation to the tree partner (Näsholm *et al.*, 2013). In contrast, N-fertilisation generally promotes a greater fraction of N transferred to the tree. Recent studies (Franklin *et al.*, 2014) have pursued to explain this variation by associating the intensity of C and N demand to the number of fungi and plants involved in the trade-off and the consequent competition for resources, similar to what occurs in a financial market. Furthermore, based on current evidence (Karst *et al.*, 2008; Jones *et al.*, 2009b), the benefit of mycorrhizal colonisation also depends on the identity of host plant (photosynthate allocation) and the wide taxonomic array of mycorrhizal fungi, hence the resulting relationships range along a spectrum from mutualism to parasitism.

The genotype variation in the cross-sectional area and the covariance of cortex area and ECM colonisation across the first three root orders, suggests all genotype roots studied were active for the uptake of nutrients. In addition, the variability in root area across genotypes changed according to growth-related traits, so the genotype with overall greater root area had also the greater root dry weight (genotype 48). In this study, the functional classification of roots by order (Pregitzer *et al.*, 2002) was especially informative, considering the variation in root area across the three genotypes studied. Studies in fine-roots of trees have reported within-species variation in root responses, influencing the lifespan of absorptive roots (e.g. McCormack *et al.*, 2014) within and between sites and across time periods. However, the study of root characteristics in response to environmental factors and the significance of this at plant level are not well understood, given the complex interaction between local and whole-plant responses.

The strong influence of N form on the measured root traits (root area, cortex-stele and the extent of ECM colonisation) provide evidence that changes in the form of N fertilisation is a primary driver of root plasticity, although links between the observed genotype variation and growth remain unclear. Little research has been undertaken on the effect of organic N in root development (Paungfoo-Lonhienne *et al.*, 2008), and previous studies in trees have indicated that organic and inorganic fertilisation differently influence root production and lifespan (Baldi *et al.*, 2010). Owen & Jones (2001) indicated this phenomenon might be due to the low diffusion of organic N, so plants and ectomycorrhizae need larger root or hyphal area to acquire organic N. Interestingly, this latter argument agrees with studies that support amino acids leading to a greater biomass allocation in roots and greater colonisation of the roots (e.g. Cambui *et al.*, 2011).

The different strength in the relationship between stele and cortex area in trees grown with L-arginine and NH_4NO_3, allow to speculate that L-arginine-treated roots might enhance root capacity for resources transportation rather than uptake, due to a greater reliance on hyphal nitrogen absorption by the fungal partner. Studies in local root responses to the effect of exogenously applied amino acids showed significantly different responses in root growth and development - either promoting or inhibiting root growth (Walch-Liu *et al.*, 2006; Forde & Lea, 2007; Forde, 2014). For example, Walch-Liu *et al.* (2006) characterised the short-term effect of L-glutamate in the root system architecture

of *Arabidopsis* in sterile conditions, and provided evidence that L-glutamate inhibits primary root growth while stimulating growth of lateral roots. It has been hypothesised that the local root responses to different amino acids might be consequence of adaptive responses for root foraging (Forde & Lea, 2007). All together, suggests that the form of N additions (organic vs inorganic N) as well as the amino acid kind differently regulate root development, both (1) through the effect of localised root responses but also (2) by the bidirectional communication between plant roots and ectomycorrhizal fungi. Nevertheless, since the present study only considered local root responses rather than architectural changes on the whole root system, it might not be appropriate to draw general conclusions about the functional significance of the morphological response.

While enhanced genotype-specific growth response to N form were found, differences in root traits appeared mainly driven by the form of N source. The observed greater growth response to L-arginine agrees with previous studies (Gruffman *et al.*, 2013; Franklin *et al.*, 2017) that have postulated that differences in growth observed between organic and inorganic N might be a consequence of energetic assimilation advantages of organic over mineral sources. The present study extends these findings and suggests the hypothesis that root response to N form promote plasticity among genotype responses. This suggests that specific metabolic strategies adopted at genotype level can benefit from the effect of N source and result in greater biomass. Whether the N use efficiency is a key determinant in the observed intraspecific variation or not, the results suggest that N source preference cannot be easily defined. Instead, it depends on a wide and dynamic range of environmental and plant physiological factors either at local and systemic level, that simultaneously overlap. Furthermore, differences in N utilisation between mineral and organic N sources might be controlled by rhizosphere soil processes that couple the C and N cycles as a result of root-microbe interactions (see Chapter 5).

2.5 Conclusions

The study revealed the presence of genotype-specific preference for N form in terms of growth, with no significant effects in N acquisition capacity. Despite the lack of clear patterns between root anatomical or biological traits and total growth, root biotic and abiotic traits clearly responded to N form. Overall, the results demonstrate that temperate

trees are able to uptake and utilise inorganic and organic N forms at similar rates, solely or in combination. The observed within-genotype variation in biomass growth suggests genotypes might possess different assimilation strategies induced by the form of the N source. Further research on root phenological changes and root lifespan will provide basic knowledge to understand how tree root systems respond to the form and rate of N applied in field conditions. Efforts should also be made to the study of adaptive strategies to available nitrogenous compounds, in order to help predict the performance of tree species across environmental gradients.

Chapter 3

Host genotype and nitrogen form shape the root microbiome of *Pinus radiata*

3.1 Introduction

It is increasingly recognised that plants associate with a diverse group of microorganisms in the rhizosphere and the root itself, which are collectively known as the root microbiome. Members of the root microbiome can interact directly and indirectly with the host plant significantly impacting plant growth and fitness (Bonfante & Anca, 2009; Berendsen *et al.*, 2012). Key determinants of root microbiome composition are edaphic factors (i.e. soil physico-chemical properties) and selection by the host (Lebeis *et al.*, 2015). Traditionally, biological processes in the rhizosphere were considered net outcomes of antagonistic and mutualistic relationships, and research efforts focused on the effect of pathogens, rhizobia and mycorrhizal fungi. However, recent research indicates that plants rely on and actively recruit a range of soil microorganisms in the rhizosphere (Lau & Lennon, 2012; Lebeis *et al.*, 2015). The relative roles of soil properties, host selection, and their interaction in shaping these complex communities are unclear.

Nitrogen (N) is an essential element for plant growth and development, and inorganic N forms (NH_4^+, NO_3^-) are typically considered the dominant available N sources. While organic N compounds, including amino acids, may contribute to plant N budgets in many ecosystems (Raab *et al.*, 1996; Näsholm *et al.*, 1998; Kielland *et al.*, 2006), we still lack direct evidence supporting the ecological significance of this capacity. Differences in N uptake capacities between and within plant species, nutrient availability, and the type and abundance of N forms, all contribute to the organic N dynamics of ecosystems (Jones & Kielland, 2002; Näsholm *et al.*, 2009). In addition, understanding the role of organic N

in plant N budget is complicated further by the fact that plant resource acquisition takes place within a multi-domain belowground ecosystem.

In conifers, ectomycorrhizal (ECM) fungi colonise tree roots, facilitating nutrient capture (mainly nitrogen and phosphorus) and water uptake in exchange for carbohydrates. There are approximately 20,000-25,000 ECM fungal species (Rinaldi *et al.*, 2008), which vary greatly both between species and among strains, particularly in their ability to access nutrients (Hodge *et al.*, 1995). ECM fungi are able to take up both inorganic and organic N forms (Talbot *et al.*, 2013) and complex organic substrates (Abuzinadah & Read, 1988; Müller *et al.*, 2007), potentially resulting in a wider resource base for the plant. Phillips *et al.* (2013) recently proposed that ECM have an organic nutrient economy with slower rates of nutrient cycling compared to arbuscular mycorrhizal fungi. Studies of plant carbon (C) investment in ECM fungi showed that plant growth benefits from improved N uptake with no additional cost for the plant (Corrêa *et al.*, 2012), but the rate of N transference by mycorrhizal fungi can be influenced by the availability of N in soil (Näsholm *et al.*, 2013). In this context, theoretical models proposed by Franklin *et al.* (2014) suggest symbiotic partners have relationships based on an *ecological market*, in which the amount of N and C transferred depends also on the number of plants and fungal partners involved in the relationship. The degree of mutualism in these relationships depends on the partners' identity, the amount of C transferred from the host and the mycorrhizal growth requirements, in addition to the environmental context (Talbot & Treseder, 2010). Therefore, variation in plant genotype response to the environment (G x E) might not only drive nutrient status but influence interactions with their mycorrhizal partners.

Plants compete with soil microorganisms for resources, although roots are considered relatively poor competitors for N (Hodge *et al.*, 2000; Kuzyakov & Xu, 2013). Recent research has shown that N depolimerisation (breakdown of N-containing polymers into small organic molecules) may be an important regulator of the terrestrial N cycle (Schimel & Bennett, 2004). Low-molecular weight compounds in plant litter (Jones & Hodge, 1999) and root exudates are N and C sources that can substantially contribute to microbial growth (Sivolodskii, 2009), potentially altering the composition and function of soil microbial communities. The importance of genotypic variation in aboveground plant traits

to soil microbial dynamics and nutrient availability is well established (Schweitzer *et al.*, 2004). Variation in root traits among plant species and genotypes can also lead to shifts in soil microbial community composition with potential implications for carbon and nutrient cycling, and soil structural stability (Bardgett *et al.*, 2014; Legay *et al.*, 2014). However, genotypes can vary in how they respond to changes in the environment, including nutrient availability, so there is potential for the interaction between tree genetics and environment (G x E) to determine the composition of microbial communities in the rhizosphere (Abdala-Roberts & Mooney, 2013).

The contribution of genotype by environment interactions to the composition of the root microbiome is unclear. Yet these interactions can have important implications for the efficiency of agricultural crops and eco-evolutionary feedbacks that drive species' fitness and adaptation. Here I address this question by characterising the root microbiome of two *Pinus radiata* D. Don genotypes with distinct growth responses to fertilisation with organic or inorganic N. Specifically I assessed (1) the influence of N form, tree genotype and their interaction on the richness, structure and composition of the root microbiome (root-associated fungi, rhizosphere fungi and bacteria) and (2) relationships between plant growth responses and the variation in the root microbiome.

3.2 Materials and Methods

3.2.1 Genotype screening and growing conditions

To select tree genotypes with distinct responses to N form, the investigation relied on a parallel study where variation in growth and physiological responses were characterised between and within ten *P. radiata* genotypes (provided by Forest Genetics Limited, New Zealand) to organic and inorganic N. 50-ml aliquots of equimolar N solutions (0.1 M) of organic (L-arginine, 0.025 M) and inorganic (NH_4NO_3, 0.050 M) N forms were applied to 12 replicate plants of each of the ten genotypes. The average biomass of the tree ramets before planting was 7.02 ± 0.83 g DW (4.43 ± 0.45 g shoot DW, 2.59 ± 0.42 g roots DW). Trees were planted individually in 4 litre pots containing standardised unfertilised potting mix (15% bark, 50% pine fines, 15% cocoa fibre, 20% pumice; see Table A.2 for details) with good drainage and aeration to encourage root growth. Pots were placed in a fibreglass

greenhouse without temperature control (averaging 20°C in Summer and 18°C in Autumn) and under natural light, located at the University of Canterbury (43°31'S, 172°35'E). For a 6-month period N treatments were applied fortnightly with N-free Ingestad solution pH 5.5 (Ingestad, 1979). Nutrients were provided at the following concentrations: 1.35 g l^{-1} N, 0.17 g l^{-1} P, 0.87 g l^{-1} K, 3.37 mg l^{-1} Ca, 5.54 mg l^{-1} Mg, 3.78 mg l^{-1} S, 169.32 mg l^{-1} Fe, 6.08 mg l^{-1} Zn, 6.35 mg l^{-1} Cu, 98.37 mg l^{-1} Mn, 0.98 mg l^{-1} Mo, 250 mg l^{-1} B, 11.48 mg l^{-1} Cl and 1.75 mg l^{-1} Na. During the experiment pots were regularly moved around the greenhouse, and tree height and root collar diameter were measured monthly. After 6-months of growth, N uptake rates were quantified for the genotypes to provide a functional basis for assessing differences in root microbiomes. Briefly, N uptake rates were determined using plants pre-treated with the corresponding organic and inorganic N fertiliser solutions 24 h before applying N solutions matching the N form applied during the growing period but containing stable isotopes; 1 atom % ^{15}N excess for the NH_4NO_3 solution ($^{15}NH_4^+$ $^{15}NO_3^-$, > 0.98% ^{15}N) and 1 atom % $^{15}N^{13}C$ excess for the arginine solution (U-[$^{13}C_6$], [$^{15}N_4$]-L-arginine, > 0.99% ^{15}N) (Persson *et al.*, 2006). Trees were harvested 24 h after the isotope addition and rates of N uptake were calculated by subtracting the natural abundances of the heavier isotopes from the atom % in each labelled sample, relative to root DW. Sample $\delta^{15}N$ values were obtained after combustion of the samples, separation by gas chromatography and analysis by continuous-flow mass spectrometry (Europa Scientific 20/20 isotope analyser, Europe Scientific, Crewe, UK) at the University of Waikato (Hamilton, New Zealand). Two full-sib genotypes were selected based on their distinct growth response to N form and the corresponding growth media and root samples from three randomly chosen replicates were used for characterising the root microbiome.

3.2.2 Processing of root and rhizosphere samples

After harvesting, root microbiomes were carefully subsampled. Specifically, fungal and bacterial rhizosphere communities were obtained from rhizosphere media samples, while fungal root-associated communities were obtained from root samples and were composed of root-inhabiting organisms, such as endophytes and rhizoplane organisms. Rhizosphere media and fine roots were collected from three replicate *P. radiata* plants of two genotypes

(known as genotypes 31 and 48). Rhizosphere media was collected by gently removing trees from the pots and obtaining the loose soil attached to roots by carefully shaking the trees to prevent mechanical root damage. Rhizosphere media samples were obtained by manually discarding any remaining roots from the soil - these were then individually homogenised and sieved (250 μm) before taking a subsample of approximately 10 g. Samples of rhizosphere media were used to extract rhizosphere DNA. After removing growth media from roots, root systems were washed in running tap water until no trace of potting media particles was evident. Approximately 8 g of fine roots ($<$ 2 mm) per tree were collected and used to extract root genomic DNA. Samples of rhizosphere media, fine roots and a representative sample of needles, were immediately frozen in liquid nitrogen. Subsamples of needles and rhizosphere media were used for N and C analysis after being homogenised, freeze-dried and pulverised with a ball mill to a fine powder. Nitrogen and C contents were analysed with an elemental analyser (Elementar Isoprime 100 analyser, Isoprime, UK). Rhizosphere media samples for pH determination were stored at 4°C no longer than 24 h, and the analysis was carried out by shaking 10 g of soil in 14 ml of 1 M KCl for 120 minutes and measuring the pH of the resulting extract (Miller & Kissel, 2010). Rhizosphere media moisture content was calculated as the percentage of water contained in samples after drying approximately 200 g of soil at 60°C for 48 h ($\pm$ 0.01 g). Fresh weight (FW) of washed roots was measured ($\pm$ 0.01 g) prior to storing a subsample of fine roots in 10% (v/v) ethanol solution at 4°C to quantify the degree of mycorrhizal colonisation by the gridline intersection method (Brundrett *et al.*, 1996). Fine roots were cut into 1-cm pieces, arranged lengthwise in a thin layer at the gridline and examined under a dissecting microscope. Approximately 300 intersections were assessed for each root sample and the proportion of ECM colonised root lengths was calculated as the percentage of intersects with mycorrhizal tips over the total number of intersects. Total leaf area was calculated by estimating the fascicle surface area (S_{fol}) of approximately 10 g of foliage FW using the formula $S_{fol} = \mathrm{dl}\ (3 + \pi)$ (Beets, 1977); with d as the diameter and l as the length of the fascicle (assuming three needles per fascicle), and finally extrapolating the total leaf area per plant by multiplying by total dry weight (DW). Dry weight partitioning of three biomass fractions (needle, stem and root) was obtained after oven-drying at 60°C for 48 h ($\pm$ 0.01 g). Composition and properties of the rhizosphere media were measured at the

end of the experiment and are displayed in Table A.2.

3.2.3 Community DNA isolation, PCR and sequencing

Root genomic DNA was isolated in triplicate from 150 mg of homogenized and finely ground material using cetyltrimethyl ammonium bromide (CTAB) buffer (3% CTAB 150 mM Tris-HCl, 2.5 M NaCl, 2 mM EDTA, 0.2% β-mercaptoethanol, pH 8). Rhizosphere DNA was isolated in a single extraction from approximately 6 g sieved potting mix using the MoBio PowerMax Soil gDNA Isolation Kit (MoBio Laboratories, Solana Beach, CA) following the manufacturer's protocol, but eluting in 500 μl Tris-HCl buffer. The concentration of total extracted DNA was measured spectrophotometrically, and samples were cleaned and concentrated (0.5 M NaCl, 100% cold isopropanol, 70% cold ethanol) or diluted using Tris-HCl buffer (pH 8) to equal concentrations. Amplicon libraries were generated for bacteria in rhizosphere DNA and for fungi in rhizosphere and root DNA. Bacterial 16S rRNA gene libraries were generated by amplifying the V3 and V4 region (approx. 460 bp) using the primers S-D-Bact-0341-b-S-17 forward (5'- CCTACGGGNGGCWGCAG -3') and S-D-Bact-0785-a-A-2 reverse (5'- GACTACHVGGGTATCTAATCC -3') (Klindworth *et al.*, 2013). For fungi, the target region was the internal transcribed spacer (ITS) region of the eukaryotic 18S rRNA gene (including ITS1, 5.8S and ITS2) amplified with the forward primer ITS1F_KYO1 (SSU) (5'- CTHGGTCATTTAGAGGAASTAA -3') and the ITS4 reverse primer (LSU) (5'- TCCTCCGCTTATTGATATGC -3') (White *et al.*, 1990; Toju *et al.*, 2012) generating an estimated amplicon lengths of 500-600 bp. Forward and reverse primers had Illumina adapters (Nextera) ligated at 5' (5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' forward, 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -3' reverse). Amplicons were obtained in 20 μl reactions containing 1X KAPA HiFi HotStart ReadyMix, 0.3 M of each primer, and 10-15 ng of DNA template. Amplification was carried out with the following thermocycling protocol: 95°C for 3 min followed by 30 cycles; 98°C for 20 sec, annealing for 15 sec, 72°C for 30 sec, and a final extension 72°C for 3 min. In order to achieve a more robust estimate of microbial diversity, the amplicons were pooled from three parallel PCR reactions using 50°C, 55°C and 60°C annealing temperatures (Schmidt et al. 2013). 300-bp paired-end runs were undertaken on an Illumina MiSeq instrument at New Zealand Genomics Limited

(NZGL, New Zealand). Copies of rRNA and ITS sequences were quantified by qPCR using the same primers but without the adapters. Standard curves were prepared by cloning purified and sequenced (Macrogene) 16S and ITS PCR products from *Escherichia coli* and *Amanita muscaria*, respectively. A pCR4-TOPO vector was used and transformed into Escherichia coli strain DH5α (Invitrogen Life Technologies; Carlsbad, CA, USA). All samples were analysed in triplicate with 15 μl reactions containing 1X SYBR Green Super Mix (ThermoFisher Scientific), 0.3 M of forward and reverse primers, and 10-15 ng of microbial gDNA template. Thermocycling parameters (RotorGene qPCR) were 95°C for 3 min, followed by 40 cycles 95°C for 30 s, 60°C for 20 s, and extension at 72°C followed by a melting curve (from 55°C to 95°C with an increase of 0.5°C every 5 s following completion of thermocycling) to verifying purity of amplicons. Fungi-to-bacteria ratio was determined by dividing fungal ITS by bacterial rRNA gene copy numbers, following Blazewicz *et al.* (2014).

3.2.4 Bioinformatic Analysis

Sequence processing was performed using the UPARSE pipeline (USEARCH v8.1.1756; Edgar, 2013). Joining of paired-end reads was possible only for the bacterial 16S rRNA gene libraries. For fungal ITS only read 1 was used due to a lack of overlap of reads 1 and 2 for many sequences which would result in underestimation of taxa with long ITS regions. For unpaired reads, a maximum expected error (*maxee*) of 1 and truncated reads at 250 bp were applied, while maxee = 1, minimum overlap of 50 bp and minimum length 400 bp was used for paired reads. OTU clustering at a 97% sequence identity was done for bacteria and fungi. Taxonomic assignment of the OTUs was completed with the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007) for prokaryotic OTUs and the UNITE database (Abarenkov et al. 2010, released in August 2015) for fungi, both with a confidence value of 0.8. To avoid OTUs that may be the result of sequencing error, only OTUs that were present in at least three samples were kept. A total of 2,062 bacterial and 552 fungal OTUs were detected across the samples. Samples from rhizosphere had an average of 1,420 bacterial and 320 fungal OTUs. Root samples had fewer fungal OTUs, a mean of 200 OTUs per sample compared to a mean of 357 fungal OTUs observed in the growth media of the same individual trees (further details provided in A.1). Accumulation

curves of OTUs for all groups of taxa plateaued, demonstrating that the bacterial and fungal diversity was adequately accounted for (Figure A.1). FUNGuild v1.0 (Nguyen *et al.*, 2016) was used to characterise fungi by functional groups, specifically saprotrophic fungi (saprotroph trophic mode) and ECM fungi (ectomycorrhizal guild).

3.2.5 Data Analysis

Statistical analysis was performed in R Studio (Version 0.99.485) with the community ecology package Vegan (Oksanen *et al.*, 2016). To test whether the *P. radiata* genotypes differed in their response to N form (tree height, diameter and volume, plant biomass, needle N content, N uptake rates and root colonisation), two-way ANOVAs were conducted after confirming data met assumptions for normality and homogeneity of variance. When the genotype by N form interaction was significant the analysis focused on how responses varied between genotypes using *lsmeans* package (Lenth, 2016) and Tukey pairwise contrasts. Linear regression was run to test for a relationship between root colonisation and diameter using 4 individual replicates. To test for differences in richness variance between N forms in rhizosphere fungal community, the homogeneity of variance was tested with Bartlett's test of sphericity. Differences in diversity between root and rhizosphere fungi were assessed using a one-way ANOVA testing for fungal community richness. The relationships between microbial richness and tree biomass were tested with Pearson's correlations on the function *cor.test*.

For each of the root microbiome communities characterised in this study (root fungi, rhizosphere fungi, and rhizosphere bacteria), the analysis tested whether OTU richness and the relative abundance of individual taxa differed between tree genotypes, N forms or for interactive effects by conducting 2-way ANOVAs, followed by one-way ANOVAs for the individual genotypes when the interaction was significant. A perMANOVA was performed to test the effect of N form, genotype and their interaction on community composition. Non-metric multidimensional scaling (NMDS) ordinations were generated to visualise relationships among bacterial and fungal communities. Mantel tests were conducted to determine the relationship between community distance matrices, estimated using Bray-Curtis dissimilarity for the relative abundance of OTUs. To determine the most influential predictor variables for each of the root microbiome communities, distance-

based redundancy analyses (db-RDA) was conducted based on the Bray-Curtis distance using the function *capscale*. The importance of each plant predictor was first assessed individually using marginal tests. To identify the model of predictors that best explained the dissimilarity in the community patterns of abundance, a stepwise procedure was used with variable selection based on adjusted R^2 (R^2_{Adj}). Predictors were added or removed from the models after testing for the inclusion of each successive predictor. Co-linearity among the environmental predictors was tested prior to analysis using *varclus* function (Hmisc package) and highly correlated variables (> 0.50) were omitted. Multiple regression analysis was used to predict tree biomass or ECM:saprotroph ratio based on the interaction between N form and ECM:saprotroph or tree biomass, respectively, using the *lm* function.

The raw sequence reads reported in this paper have been deposited in the National Center for Biotechnology Information database (submitted in the Sequence Read Archive as individual fastq files per experimental unit; SRA accession SRP077412; BioProject PRJNA201483, accessions SAMN05300207 - SAMN05300230).

3.3 Results

3.3.1 *Pinus radiata* intraspecific variation in response to nitrogen form

I first quantified how the two *P. radiata* genotypes responded to organic and inorganic N. Genotypes 31 and 48 differed in their aboveground biomass allocation in response to organic versus inorganic N, while tree height and diameter were not affected (Table A.1). Two-way ANOVAs indicated significant interactions between N form and tree genotype for aboveground biomass measures (Stem: $F_{(1,35)} = 4.39$, $p = 0.04$; Foliage, $F_{(1,35)} = 6.39$, $p = 0.02$), likely contributing to the interactive effect on total biomass ($F_{(1,35)} = 4.82$, $p = 0.03$). Then, I assessed growth responses to the different N forms for each genotype individually. For genotype 31, organic N resulted in greater overall aboveground biomass than for trees supplied with inorganic N. In contrast, genotype 48 exhibited no differences in growth in response to organic and inorganic N sources (Figure 3.1a, Table A.1). There was not an interactive effect of tree genotype and N form for needle N content (%), although there was a significant effect of N form alone ($F_{(1,35)} = 4.25$, $p = 0.04$). Needle N content was significantly greater in both genotypes when L-arginine was supplied (Figure 3.1b, Table

A.1). No significant differences between genotypes were found for belowground biomass allocation or N uptake capacity (Table A.1).

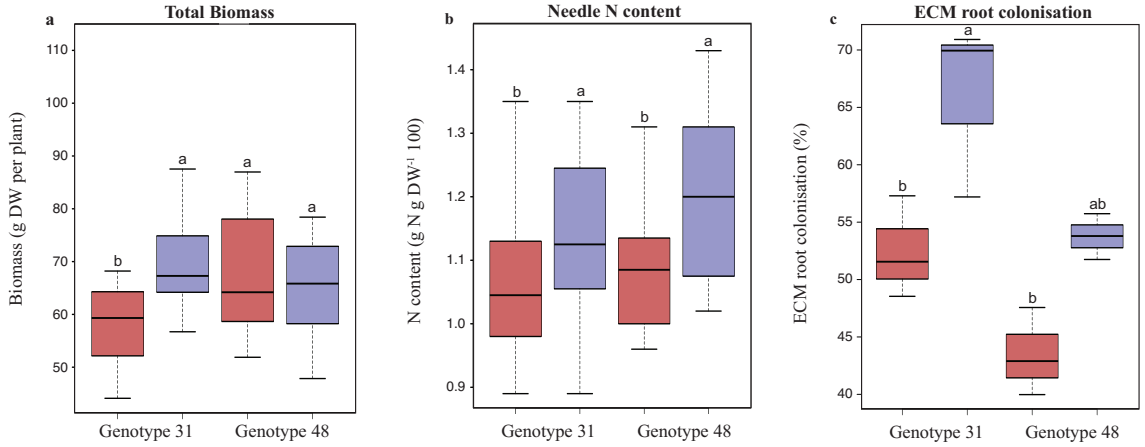

Figure 3.1: Boxplots for (a) total tree biomass, (b) foliage N content and (c) ECM root colonisation for genotypes 31 and 48 in response to inorganic N (red boxes) and organic N (purple boxes) supply. The bottom and top edges of the boxes mark the first and third quartiles, the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above bars indicate significant ($p < 0.05$) plant responses to genotype by N form interaction (for a) and to main N form effect (for b and c) using 2-way analysis of variance followed by a *post hoc* Tukey pairwise comparison.

3.3.2 Root colonisation

Rates of root colonisation by ectomycorrhizal (ECM) fungi were not influenced by an interaction between N form and genotype ($p > 0.05$), although ECM colonisation rate differed significantly between both tree genotypes ($F_{(1,35)} = 13.95$, $p = 0.006$) and N forms ($F_{(1,35)} = 17.57$, $p = 0.003$). Colonisation levels were greater for genotype 31 than for 48, and greater in L-arginine than NH_4NO_3 with 20% greater colonisation in the organic N treatment (Figure 3.1c, Table A.1). Correlations to test whether the relationship between colonisation rate and root diameter was influenced by N form or genotype across 4 individual replicates, revealed significant negative correlations indicating that regardless of the N form, there is a consistent decrease in colonisation with thicker fine roots (Figure 3.2).

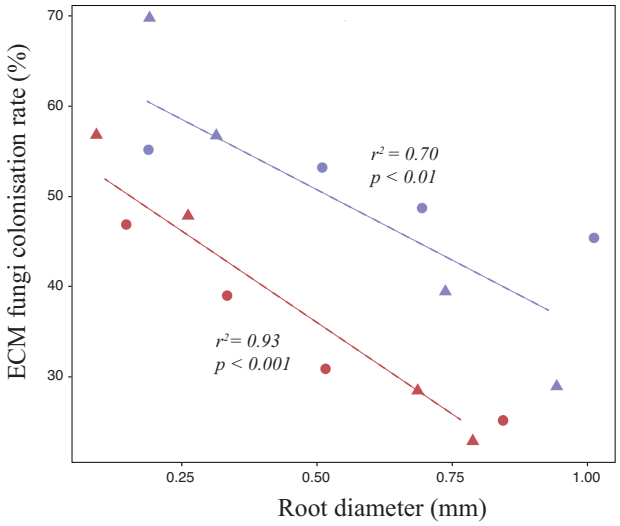

Figure 3.2: Strength of linear relationships between the degree of colonisation by root ECM fungi (%) and fine root diameter (mm) in genotype 31 (triangles) and 48 (circles) fertilised with inorganic N (red) and organic N (purple). Points represent mean values of approximately 300 root intersections in each of four individuals assessed per combination of genotype and N form.

3.3.3 Root fungal community responses to nitrogen form and tree genotype

The number of fungal OTUs detected in root samples was influenced by a significant interaction between N form and tree genotype ($F_{(1,8)} = 14.32$, $p = 0.004$). When the impact of N form was assessed for each genotype individually (Figure 3.3a), more OTUs were observed when trees were fertilised with NH_4NO_3 than with L-arginine for genotype 31 ($F_{(1,5)} = 41.81$, $p = 0.003$). In contrast, for genotype 48 there was no difference between N forms in the number of fungal OTUs colonising roots ($F_{(1,5)} = 0.03$, $p = 0.865$). There was also a significant interaction between N form and tree genotype on the overall composition of the fungal community in root samples (Figure 3.4a, Table 3.1). *Pinus radiata* root fungal communities were dominated by members of phyla Ascomycota (classes Sordariomycetes and Eurotiomycetes), Basidiomycota (Agaricomycetes, Tremellomycetes) and Chytridiomycota (Chytridiomycetes). The relative abundance of Eurotiomycetes was significantly reduced under organic N in both genotypes (N form: $F_{(1,8)} = 13.69$, $p = 0.006$), while Sordariomycetes was influenced by a N form by tree genotype interaction that promoted a greater abundance in genotype 31 when organic N was supplied (Genotype x N form: $F_{(1,8)} = 11.87$, $p = 0.008$) (Figure 3.6).

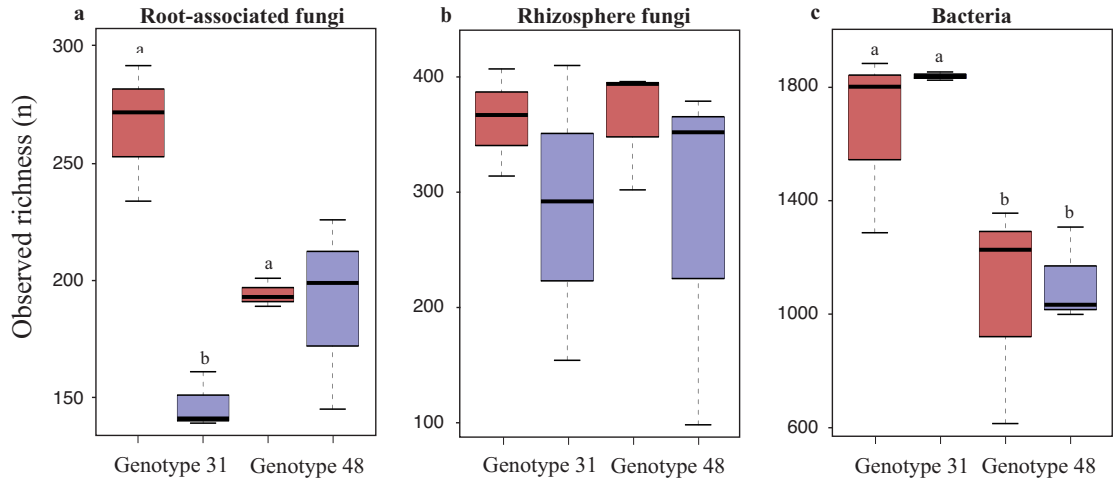

Figure 3.3: Observed richness for (a) root-associated fungi, (b) rhizosphere fungi and (c) rhizosphere bacteria in the root microbiome of genotypes 31 and 48 with inorganic (red) and organic (purple) N fertilisation. The bottom and top edges of the boxes mark the first and third quartiles, the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above boxplots indicate significant differences ($p < 0.05$) based on a *post hoc* Tukey pairwise comparison.

Root fungal community responses were also assessed in terms of fungal guilds. Interactive effects of N form and genotype significantly influenced the relative abundance of ECM ($F_{(1,8)} = 10.64$, $p = 0.011$) and saprotrophic ($F_{(1,8)} = 13.10$, $p = 0.006$) fungal guilds (Figure 3.5). When genotypes were analysed individually, the relative abundance of ECM was significantly influenced by N form in genotype 48 ($F_{(1,4)} = 9.37$, $p = 0.03$) with an increase in the organic N as compared to the inorganic N, but no significant differences in ECM were observed for genotype 31. In contrast, saprotrophs were more abundant in genotype 31 with inorganic N supply than in the organic N supply ($F_{(1,4)} = 27.5$, $p = 0.006$), but there was no difference for genotype 48.

Table 3.1: Results of perMANOVA (permutational MANOVA) for the impacts of host genotype, form of N supply and the interaction between host genotype and N form on the composition of the root microbiome communities in two genotypes of *P. radiata*. The results were obtained using Bray-Curtis dissimilarity and 9999 permutations.

	N form			**Tree genotype**			**G x N**		
	F Model	R^2	*p*	F Model	R^2	*p*	F Model	R2	*p*
Root fungi	2.189	0.157	**0.041**	0.834	0.060	0.588	2.900	0.208	**0.022**
Saprotrophic fungi	2.871	0.196	**0.001**	1.754	0.119	0.075	1.999	0.137	0.052
ECM fungi	2.054	0.150	0.121	0.384	0.027	0.818	3.292	0.239	**0.028**
Rhizosphere fungi	0.701	0.070	0.728	0.677	0.067	0.749	0.670	0.067	0.755
Bacteria	5.248	0.285	**0.001**	3.030	0.164	**0.002**	2.146	0.116	**0.042**

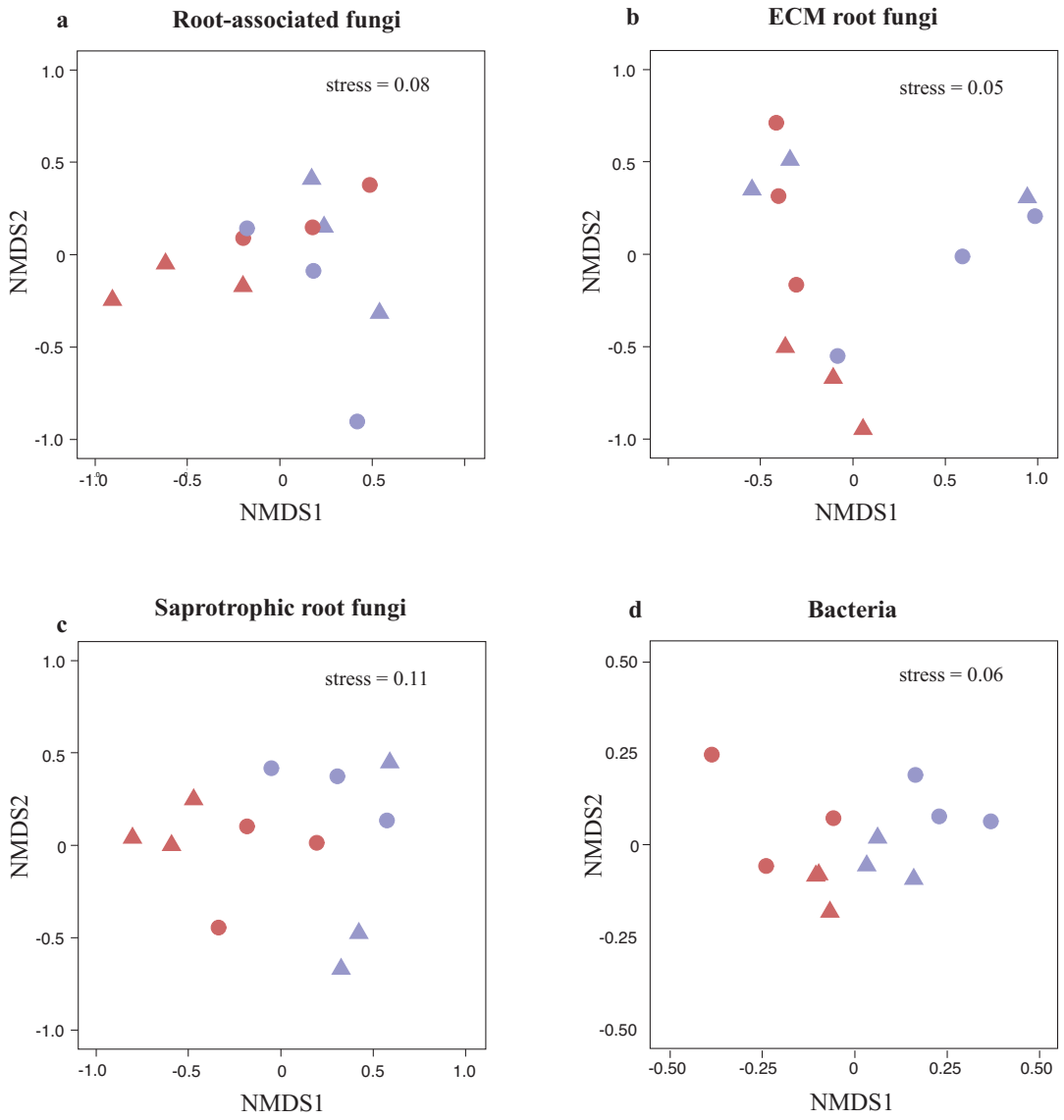

Figure 3.4: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity for (a) root-associated fungal, (b) ECM root fungal, (c) saprotrophic fungal and (d) bacterial composition. Points represent samples from genotype 31 (circles) and 48 (triangles) in response to inorganic N (red) and organic N (purple) fertiliser. Stress values are displayed in each of the ordinations.

ECM and saprotrophic richness were also significantly impacted by interactive effects. Significantly greater ECM richness was observed in response to inorganic N than in the organic supply for genotype 31 ($F_{(1,4)} = 16.2$, $p = 0.015$), but no difference in genotype 48 (Table 3.2). Similarly, the effect of N form on saprotrophic richness was only significant in genotype 31 ($F_{(1,4)} = 52.9$, $p = 0.001$), in which inorganic N had more fungal OTUs than organic N. Finally, the ratio between ECM and saprotrophic fungi (ECM: saprotrophs) was significantly affected by N form ($F_{(1,8)} = 6.02$, $p = 0.039$) and not impacted by an interaction between N form and tree genotype ($F_{(1,8)} = 1.22$, $p = 0.300$). The roots of trees treated with L-arginine had a significantly greater ECM-to-saprotroph ratio (8.76 ± 5.70) than roots treated with NH_4NO_3 (2.90 ± 0.90) across genotypes. When the ectomycorrhizal (ECM) and saprotrophic members of the root fungal communities were considered separately, I also observed a significant interaction between N form and tree genotype for the composition of the root ECM community (Figure 3.4b, Table 3.1). In contrast, composition of the root saprotrophic fungal community was influenced by N form but not by tree genotype (Figure 3.4c, Table 3.1).

3.3.4 Rhizosphere fungal community responses to nitrogen form and tree genotype

Richness of the rhizosphere fungal community was not influenced by N form or genotype (Genotype x N form: $F_{(1,8)} = 0.007$, $p = 0.935$; N form: $F_{(1,8)} = 1.795$, $p = 0.217$; Genotype: $F_{(1,8)} = 0.004$, p = 0.952) (Figure 3.3b). Overall, rhizosphere fungal communities showed greater diversity than the root-associated communities ($F_{(1,22)} = 14.31$, $p = 0.001$). Variation in fungal species richness differed between N forms (Bartlett's test, ($\chi^2_{(1)} = 4.22$, $p = 0.030$). Rhizosphere soil treated with L-arginine had a greater range of fungal species richness between samples, while the number of fungal species detected in the rhizosphere of inorganic N treated trees was more consistent. Composition of the rhizosphere fungal community was not impacted by N form, genotype, or an interaction (Table 3.1). Rhizosphere fungal communities were dominated by similar taxa as the root-associated communities, and the most abundant fungal orders shared between rhizosphere and root sample types were *Xylariales* and *Polyporales*, neither of which are members of ECM fungal guild. (Figure A.2). However, the relative abundance of Ascomycota in the

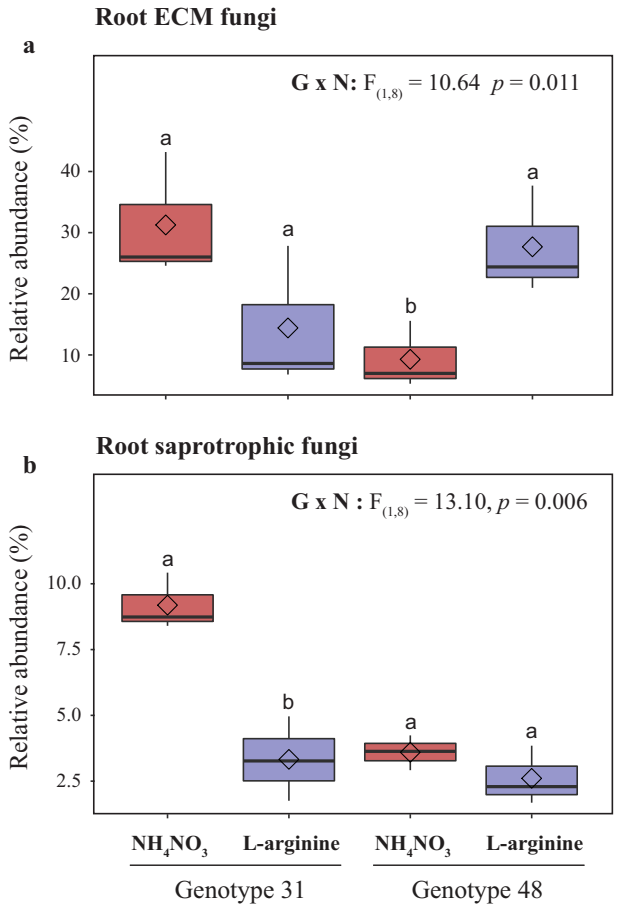

Figure 3.5: Relative abundances of (a) ectomycorrhizal fungi and (b) saprotrophic fungi in the root microbiome communities of *P. radiata* genotypes fertilised with inorganic N (NH_4NO_3, red) and organic N (L-arginine, purple). Rhombus and sectional lines in boxplots indicate mean point and averaged values (n = 3), respectively.

rhizosphere was consistently lower than in the root samples ($F_{(1,22)} = 9.35$, $p = 0.005$), possibly leading to the lower Ascomycota-to-Basidiomycota ratio in rhizosphere (0.43 ± 0.14) than in root samples (1.08 ± 0.28). The lack of correlation between distance matrices for root-associated and rhizosphere fungal communities (Mantel test; $r_M = 0.01$, $p > 0.05$) suggests composition of root and rhizosphere fungal communities is determined different factors.

Table 3.2: Observed richness (mean with one standard deviation) of ectomycorrhizal and saprotrophic root-associated fungal communities in genotype 31 and 48 of P. radiata fertilised with inorganic N (NH_4NO_3) and organic N (L-arginine). Columns on the right indicate the significance of N form and N x G interactive effects of the observed means. Main genotype effect was insignificant for both communities.

	Genotype 31		**Genotype 48**		**N**	**G x N**
Root communities	Inorganic N	Organic N	Inorganic N	Organic N	$F_{(1,8)}$ (p)	$F_{(1,8)}$ (p)
ECM fungi richness	18.7±2.5	12.7±0.6	18.3±0.6	17.7±2.5	10.0 (< 0.05)	6.4 (< 0.05)
Saprotrophic richness	110.0±11.1	59.3±4.6	78.7±4.9	70.7±24.5	13.4 (< 0.01)	7.1 (< 0.05)

3.3.5 Rhizosphere bacterial community responses to nitrogen form and tree genotype

Richness of the rhizosphere bacterial community was not affected by an interaction between N form and genotype ($F_{(1,8)} = 0.186$, $p = 0.677$) (Figure 3.3c). However, there was a significant difference between genotypes ($F_{(1,8)} = 17.94$, $p = 0.002$) with greater bacterial diversity in the rhizosphere of genotype 31 than genotype 48. There was a significant interactive effect of N form and tree genotype on the composition of the rhizosphere soil bacterial community (Figure 3.4d, Table 3.1). Proteobacterial classes α-Proteobacteria (Rhizobiales) and β-Proteobacteria (Burkholderiales) dominated the rhizosphere bacterial communities, followed by Planctomycetia, Verrumicrobia Subdivision 3, Bacteroidetes, Actinobacteria, Acidobacteria classes (Gp1, Gp6, and Gp3) and Sphingobacteria. While no interactive effects were detected on the relative abundance of individual rhizosphere bacterial taxa, there were a range of responses to the main effects of tree genotype and N form (Figure 3.6, Figure A.3). Organic N increased the relative abundances of Acidobacteria Gp1 (N form: $F_{(1,8)} = 12.63$, $p = 0.007$) and β-Proteobacteria (N form: $F_{(1,8)} = 39.52$, $p < 0.001$), in contrast to δ-Proteobacteria that had greater relative abundance in the inorganic N treatment (N form: $F_{(1,8)} = 10.17$, $p = 0.012$). There were significant effects of both N form and tree genotype on the abundance of Planctomycetia (N form: $F_{(1,8)} = 26.10$, $p < 0.001$; Genotype: $F_{(1,8)} = 9.78$, $p = 0.014$), and γ-Proteobacteria (N form: $F_{(1,8)} = 12.90$, $p = 0.007$, Genotype: $F_{(1,8)} = 19.26$, $p = 0.002$). The fungi-to-bacteria ratio in the rhizosphere obtained by qPCR showed no difference across samples ($p > 0.05$, data not shown).

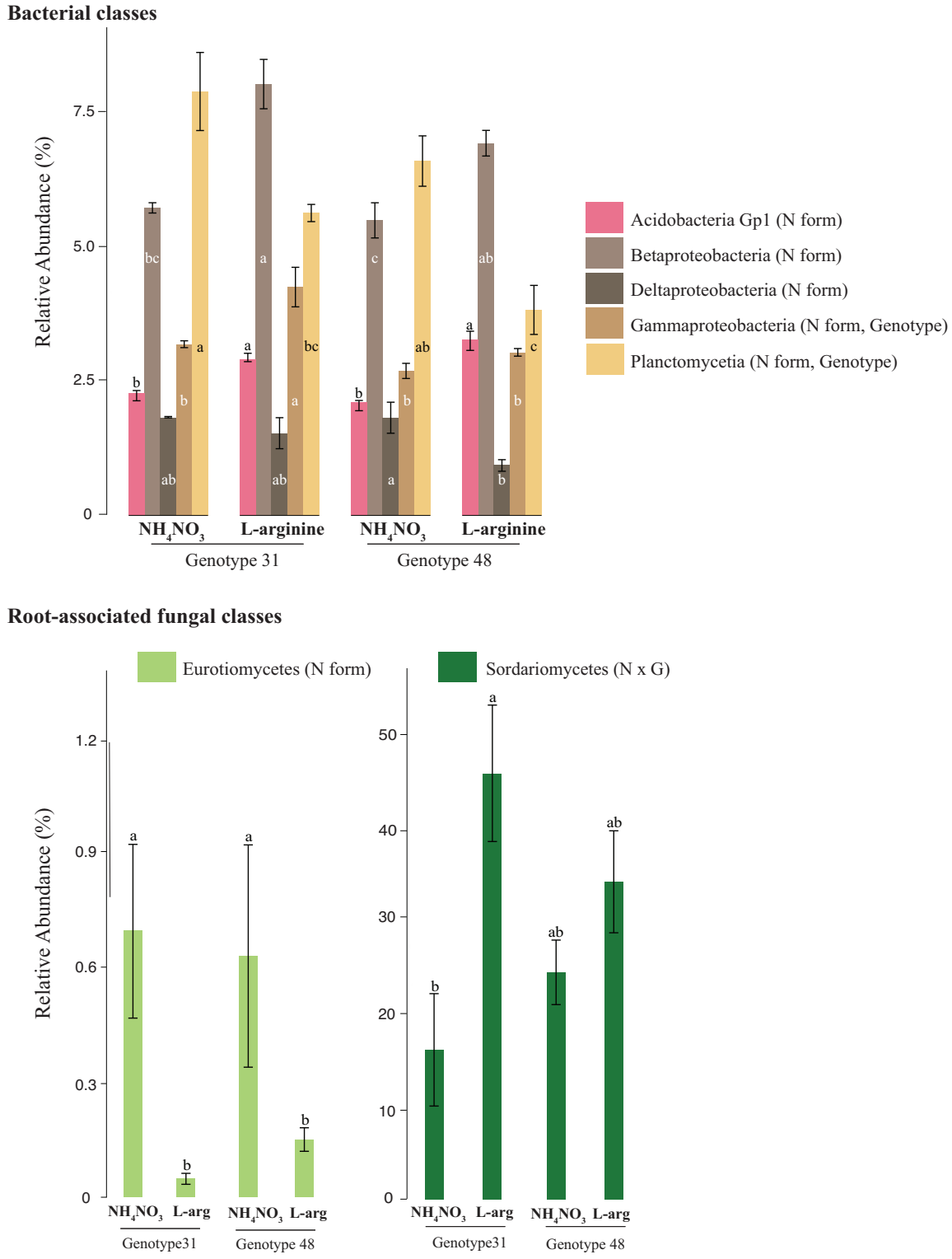

Figure 3.6: Mean relative abundances of the selected bacterial and fungal classes in the root microbiome of genotypes 31 and 48 fertilised with organic (L-arginine) or inorganic (NH_4NO_3) N fertilisation. Error bars represent standard error. Letters indicate significant differences ($p < 0.05$) based on a *post hoc* Tukey pairwise comparison.

3.3.6 Relationships between plant responses and microbial communities

To determine how N form impacted relationships between the plant host and the root microbiome, I first assessed whether there was a relationship between the root-associated fungal community and rhizosphere bacterial community, which were both significantly influenced by interactions between tree genotype and N form. No relationship was found for either diversity (Pearson's correlation: $r_{(10)} = 0.02$, $p = 0.95$) or community composition (Mantel test: $p > 0.05$) between the rhizosphere bacteria and root-associated fungi, suggesting genotype-specific responses influenced these communities in different ways. Correlations between plant measures and diversity of the root microbiome showed a trend towards an overall negative correlation between the number of root-associated fungal OTUs and plant biomass ($r_{(10)}$ = -0.50, $p = 0.09$). When this relationship was assessed for the genotypes individually, a similar trend was observed for genotype 31 ($r_{(4)}$ = -0.75, $p = 0.08$) but not for genotype 48 ($r_{(4)} = 0.38$, $p = 0.44$). In contrast, I found no relationship between richness of rhizosphere fungi ($r_{(4)}$ = -0.60, $p = 0.20$) or bacteria ($r_{(10)}$ = -0.60, $p = 0.20$) and tree biomass.

Distance-based redundancy analysis (db-RDA) was carried out to assess relationships between plant and rhizosphere soil measures and the composition of the root microbiome. Constrained analysis of principal coordinates (Bray-Curtis distance) revealed that variation in each subset of the root microbiome (root fungi, rhizosphere fungi and rhizosphere bacteria) is best explained by a different subset of plant variables (listed in Table A.1). Root dry weight ($F_{(1,9)} = 2.69$, $p = 0.020$) and needle N ($F_{(1,9)} = 1.95$, $p = 0.089$) generated the best predictor model of of the root-associated fungal composition with over the 19% of variance explained (adjusted R^2). The best predictor model of the rhizosphere fungi composition included the total leaf area ($F_{(1,9)} = 2.65$, $p = 0.013$) and needle N-to-C content ratio ($F_{(1,9)} = 2.15$, $p = 0.044$) with 20% of variance explained. The composition of the rhizosphere bacteria was significantly predicted by plant capacity to acquire N (N uptake rate) ($F_{(1,9)} = 2.26$, $p = 0.031$), with over the 10% of the variance explained.

I assessed whether variation in the relative proportion of fungal guilds in roots was associated with the differences in tree growth. A linear regression model was conducted to test whether the interaction between tree biomass and N form predicted the ratio between

ECM and saprotrophic fungi (Table 3.3), showing a significant relationship (full model: $F_{(3,8)} = 13.24$, $p = 0.002$). Correlations between total biomass and ECM:saprotroph relative abundance (Figure 3.7) revealed a significant negative relationship with organic N ($r_{(4)} = -0.85$, $p = 0.02$) and a trend for the inorganic N supply ($r_{(4)} = -0.75$, $p = 0.08$), while the relationship between the ratio of fungal guilds and total biomass did not differ between tree genotypes.

Table 3.3: Multiple linear regression model of the effect of tree biomass in response to N form on the ectomycorrhizal-to-saprotrophic fungal ratio in the root-associated fungal community. β estimates the standardised regression coefficients for the explanatory variables, N form (L-arginine, NH_4NO_3) and tree biomass.

	β	**SE**	**t value**	***p***
Tree biomass	-0.30	0.079	-3.81	0.005
L-arginine	19.42	5.52	3.52	0.007
Tree biomass:L-arginine	-0.23	0.08	-2.92	0.019
Adjusted R^2		0.77		
F		13.24		

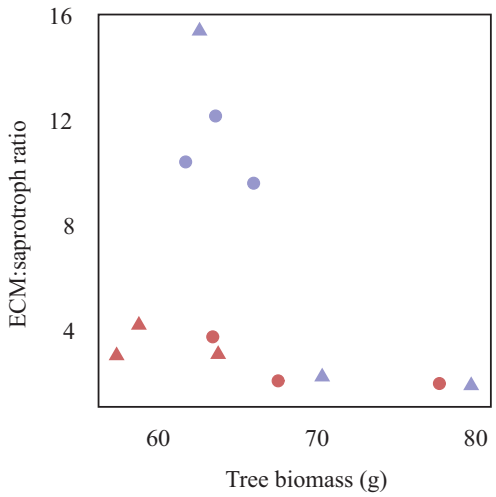

Figure 3.7: Relationships between the ectomycorrhizal-to-saprotrophic fungal ratio (ECM: saprotroph) and the total tree biomass. Points represent samples obtained from genotype 31 (circles) and 48 (triangles) in response to inorganic N (red) and organic N (purple) fertiliser.

3.4 Discussion

The results show that *P. radiata* genotypes have distinct growth responses to organic and inorganic N supply, and that these genotype-specific growth responses to N form extend to the root microbiome. Nitrogen form-driven phenotypic plasticity in biomass allocation was found in genotype 31, while no differences were found for genotype 48 when grown with organic versus inorganic N supply. This genotype by environment (G x N) response to N form is remarkable considering the limited trait variation expected between genotypes that share full ancestry (full-sib). Other studies of early growth stages show plant responses to equimolar organic and inorganic N forms can lead to changes in biomass partitioning, pressumably driven by differences in N availability between N forms (Kruse *et al.*, 2010; Cambui *et al.*, 2011). Here I expand on these findings with 1-year-old trees that have well-developed root systems. Rapid plant genotype-specific plastic responses are commonly observed in response to nitrogen limitation and drought (e.g. Corcuera *et al.*, 2012) due to variation in resource use efficiency and biomass allocation (Poorter *et al.*, 2012; Abenavoli *et al.*, 2016). These responses are important determinants of tolerance to environmental and climate change, and contribute to adaptation to novel environments (Nicotra *et al.*, 2010). For instance, intraspecific variation in response to light, temperature and CO_2 altered leaf traits, including photososynthetic rates, affecting growth. Despite the lack of knowledge about the mechanisms by which environmental signals are perceived, studies have clearly demonstrated that genotype responses driven by environment have an important impact on fundamental ecosystem processes (Whitham *et al.*, 2006), such as leaf litter decomposition and N mineralization, processes intimately linked to belowground microbial communities (Urbanová *et al.*, 2015).

Both root-associated fungal and rhizosphere bacterial communities showed G x E responses in measures of composition, although correlation between distance matrices (Mantel test) suggested that these communities have distinct responses to N dynamics. In contrast, the rhizosphere fungal community did not respond to the host genotype or N form. Bulgarelli *et al.* (2013) proposed a two-step selection model to understand assembly of root microbiome that starts with the influence of edaphic factors, rhizodeposits and cell wall features followed by genotype-dependent selection of microbes within root struc-

tures. Considering that the root-associated fungal community might be a subset of the rhizosphere community, the composition of root-associated fungal community is likely the result of active or passive selection of particular rhizosphere fungal taxa by the host plant. My findings support the hypothesis that feedbacks from host genotype presumably evolve as important factors determining composition of the root microbiome (Schweitzer *et al.*, 2008b; Wagner *et al.*, 2016). Recent studies in Arabidopsis (Lundberg *et al.*, 2012) demonstrated that specific bacterial taxa in the rhizosphere were genotype-dependant, despite soil factors explaining overall variation to a greater extent. In other studies, plant species select microbes for the root zone, presumably by creating a zone enriched in C, shaping the root microbiome to greater extent than fertilisation does (da Costa *et al.*, 2013; Ridl *et al.*, 2016). Genetic based-interactions among species are complex and have been recently gaining attention (Whitham *et al.*, 2006). The expression of genotype-specific traits affecting the communities of associated organisms, including belowground microbial communities, can have far-reaching consequences (Sthultz *et al.*, 2009). For instance, Gehring *et al.* (2014) found a complex plant genetics-based interaction with the ECM community, further influenced by susceptibility to herbivory and drought tolearance, determining plant interspecific competiton.

While it is not possible to untangle direct responses of the root microbiome to N addition from indirect effects that are a consequence of plant growth responses to N form, the abundance of some microbial taxa did respond to N form alone. In recent studies (Zhang *et al.*, 2014), exogenous N supply signficantly increased the relative abundance of certain taxa, including Acidobacteria, Proteobacteria and Eurotiomycetes fungi. I speculate organic and inorganic N supply favoured specific rhizosphere bacteria based on their metabolic requirements. L-arginine (C_6:N_4) provides a labile C source that could stimulate growth of taxa able to outcompete others for this resource. This may explain why I observed positive responses from β and δ-proteobacteria to L-arginine supply, as these taxa have been shown to respond to C addition in other studies (Eilers *et al.*, 2010). In addition, greater ECM colonisation was observed in response to the organic N supply, which has been reported as the prefered form of N for ECM fungi (Talbot & Treseder, 2010). Changes in the root microbiome due to direct effects of the N form are likely to feedback to plant performance by altering nutrient availability, particularly N cycling rates.

One of the most intriguing results of this study is that the tree genotype whose growth responses differed between N forms (genotype 31), also had the most divergent root microbiome responses to N form. Specifically, the greater biomass in genotype 31 under organic N supply was coupled with lower richness of root-associated fungi and fungal guilds, compared to the inorganic N supply. This is further supported by the negative correlation between root fungal richness and plant biomass for genotype 31. The results are supported by recent studies linking the composition of the root microbiome with changes in tree productivity (Bazghaleh *et al.*, 2015). The observed genotype-specific shifts in the root microbiome may be explained by distinct plant responses to N form. Although the two genotypes considered here had similar belowground biomass allocation and N uptake capacities, root traits such as the composition and/or rate of root rhizodeposition can vary in response to N form and/or N form-driven plant status (Högberg *et al.*, 2010) and modify the composition of the root microbiome (Haichar *et al.*, 2008; Shi *et al.*, 2011). This scenario further supports the different relationships between plant measures (above- and belowground) and the associated microbial community responses, and suggests that the influence of N form in tree genetics drives the co-variation of plant productivity and the modulation of root C release.

The contribution of root fungal guilds, such as ECM and saprotrophic fungi, can play a role moderating the genotype-specific growth responses to N form. This agrees with the observed variation in ECM and saprotrophic species abundance and richness driven by tree genotype. The observed changes in relative abundance of ectomycorrhizal and saprotrophic fungi suggest a non-overlapping preference for N substrates, as suggested by Talbot *et al.* (2013). Although I would expect a consistent increase of ECM fungi relative abundance in response to organic N (Talbot & Treseder, 2010), considering the L-arginine-driven quantitative increase in colonisation rates, this was only observed in genotype 48. Likewise, ECM species richness was consistently greater in the inorganic N treatment than the organic N treatment. This agrees with Velmala *et al.* (2013), who found a negative correlation between the N concentration in needles and ECM richness, suggesting young trees under controlled conditions can benefit from colonisation with fewer ECM species. As postulated by Talbot & Treseder (2010), variation in host C supply due to fertilisation can affect ECM diversity and the degree of mutualism, sometimes turning toward parasitic.

Therefore, the genotype-specific plant N status driven by N form can differently influence the associated root symbiotic communities.

3.5 Conclusions

In summary, the study indicates *P. radiata* genotypes can differ in their response to N form, particularly in biomass growth. Furthermore, I demonstrate that this intraspecific variation drives changes in the root microbiome induced by the interactive effect of host genotype and N environment (G x E). While the controlled plant growing conditions minimised the effects of variable edaphic factors and plant interspecific competition, my results clearly demonstrate that differences in genotype performance in response to N form are associated with shifts in root-associated fungi and the rhizosphere bacterial community. In addition, plant above- and belowground descriptors can predict shifts in the root microbiome communities. Future research efforts should target the interdependence between host identity and associated microbiomes in forest ecosystems, in order to better understand plant-soil feedbacks as well as incorporate microbiome community ecology into forest management and agriculture practices. These results contribute to the understanding of the relationships between tree genetics and the associated microbial communities in response to organic and inorganic N forms, and highlight the potential for host genotype by environment interactions to shape the composition of host-associated microbial communities.

Chapter 4

Field-scale variability in site conditions and seasonal differences in climate explain genotypic variation in response to nitrogen source in *Pinus radiata* D. Don

4.1 Introduction

Given the longevity of sessile plants, phenotypic plasticity enables trees and other perennial woody plants to sense, change and adapt their growth and physiological responses to cope with changes in environmental conditions (Bradshaw, 1965; Corcuera *et al.*, 2010). A current challenge is to clarify which biological traits are manifested as genotypic variation from evolutionary/breeding history (G), phenotypic response to environmental variability (E) or combinations of both (G x E). There is abundant evidence that supports the idea that plant species and population responses differ across different environments (e.g. Schlichting & Levin, 1984; Valladares *et al.*, 2000) but also that genotype-specific variation can be exhibited when grown in comparable conditions, affecting adaptive capacity and consequently, genotype performance. The flexibility and adaptability of tree genotype requires special attention, not only to understand the complexity of traits in breeding programmes but also for the greater ecological impact that the increasing inter-seasonal climate changes may have in long-lived organisms.

Pinus radiata D. Don is the most common softwood plantation species growing in temperate zones of the southern hemisphere (Richardson, 1998). Plantations in New Zealand now occupy 1.7 million ha and this is the basis of a major export industry for the country (Forest Owners Association, 2016) (Figure 4.1a). Since the 1960s, the New Zealand forestry industry has undertaken multi-generation breeding programmes using

plant material from just a few provenances (Burdon *et al.*, 2008). A number of studies of *P. radiata* families have determined marked genotype variation in growth rates along nutritional gradients (Hawkins *et al.*, 2010; Xue *et al.*, 2013), suggesting that genotype-specific physiological traits might be advantageous depending on the nutritional characteristics of the site.

In situ experimental studies enable us to gain an understanding of how plants acclimate to complex environments, including aboveground and belowground community interactions. Field trials typically divide a site into homogeneous units and treatment blocks. However, this kind of blocking typically relies on a visual inspection of the site and is made with relatively little or no information of within-field soil spatial variability. Heterogeneity in soil comprises of changes in soil physico-chemical properties and associated microbial processes, which determine the capacity to hold, provide and cycle nutrients (i.e. nitrogen, phosphorus) and the water available. These are essential drivers of site quality that directly influence plant growth rate and crop yield (Landsberg, 1986). Recent development of portable, non-invasive instruments, such as the electromagnetic induction meter (EM) and the estimates of soil apparent electrical conductivity (EC_a), have made it possible to measure spatio-temporal variability in soil physicochemical properties in a time and cost efficient way (e.g. Williams & Baker, 1982; Khakural *et al.*, 1998; Stadler *et al.*, 2015). The monitoring of soil indicators in experimental and plantation sites contributes to a better understanding of local productivity by facilitating site-specific management (Kitchen *et al.*, 1999) for a sustainable forestry.

In order to achieve the genetic potential of the tree, intensive forest management incorporates silvicultural operations, including nitrogen (N) fertilisation and the control of competing vegetation (Mead *et al.*, 1984), often reduced to a set of practices that meet basic tree requirements. However, the addition of inorganic N inputs to obtain good commercial productivity rates does not necessarily match plant capacity to retain N. This provides the potential for nutrient leaching which may result in financial loss and negative environmental outcomes [e.g. eutrophication of aquatic ecosystems and a negative impact on biodiversity (Hautier *et al.*, 2009)]. In the context of sustainable productivity, it is increasingly recognised that there is a need for genotype selection based on nitrogen use efficiency (NUE) which combines firstly, the fraction of added N taken

up by the plants, and secondly, the N productivity (growth rate per plant N) (Xu *et al.*, 2012). A large body of research has investigated the effects of N availability on plant development, biomass partitioning and regulation of root nutrient uptake (e.g. Burger, 2009). However, little information is available on the ability of different genotypes to acquire, recycle and assimilate nutrients in conifers with industrial interest, such as *P. radiata* (e.g. Burdon & Shelbourne, 1971; Burdon, 1976; Hawkins *et al.*, 2010). More broadly, the relationship between genotype-specific strategies of resource use and growth rates is not well understood.

Species vary significantly in their preference for different N source (Britto & Kronzucker, 2013; Boczulak *et al.*, 2014), yet the effect of different N chemical forms in genotypes of the same species has not been directly addressed. Under controlled conditions, plants supplied with organic N have shown differences in biomass partitioning by increasing the root:shoot ratio compared with those supplied with inorganic N forms (e.g. Cambui *et al.*, 2011; Franklin *et al.*, 2017). However, plant response to different forms of N in field studies are not so clear, given the highly variable environmental conditions and the potential changes in N mineralisation rates due to shifts in rhizosphere microbial activities (Jones & Kielland, 2002). Our understanding is not helped by the small number of related studies which have been performed (e.g. Näsholm *et al.*, 2000; Gruffman *et al.*, 2012). In addition, the potential effects on the overall growth and allocation differences in response to N source are not well understood, and might ultimately depend on additional genetic and environmental components. Little is known about the natural origin of species divergences in N form use, and whether this reflects adaptation to the most abundant source of N in the natural habitat (Pearson *et al.*, 1989; Kronzucker *et al.*, 1997) or niche separation to reduce N competition among plant species (McKane *et al.*, 2002). Although comparisons of N uptake rates show organic and inorganic N forms may be taken up to a similar extent (Falkengren-Grerup *et al.*, 2000; Öhlund & Näsholm, 2001; Finzi & Berthrong, 2005), recent empirical studies provide evidence that organic N might lead to greater nitrogen efficiency compared to inorganic N. This is due to a reduction in the C cost of organic N molecules (C bonus) (Franklin *et al.*, 2017) and the mismatch between N availability and the capacity of plants to acquire inorganic N (Brackin *et al.*, 2015).

Nutrient acquisition is strongly influenced by water availability. In addition to the

impact of soil water content on microbial activity and nutrient mineralisation rates, transpiring plants influence the mass flow of water from the soil solution towards the root surface, while the movement of water facilitates nutrient transfer (Chapin *et al.*, 1988). An increase in the concentration gradient between the root surface and bulk soil drives the diffusion of compounds towards roots. Diffusion coefficients depend on molecular size which suggests transpiring plants may access variable contributions of N sources. In soil, amino acids have generally lower diffusion coefficients than inorganic N forms (e.g. NO_3^-), and this might increase the chance of organic N sources being consumed by soil microbes rather than plants (Miller & Cramer, 2004). It is broadly accepted that the effect of N-fertilisation induces an increase in leaf area and leaf photosynthetic rates that promotes growth. However, an increase in plant productivity also requires an increase in the potential for transpiration and water use. In conditions of water deficit, early plant responses trigger stomatal closure in order to prevent cell and tissue dehydration, xylem cavitation and potential death. Because of this, water stress can minimise the positive effects of fertilisation on growth, through the reduction of gas exchange in leaves. Given the changes in water dynamics predicted for global climate change (Basher, 2000), research on the interactive effect of fertilisation and water stress on growth and ecophysiological processes is increasingly gaining attention (Samuelson *et al.*, 2014). These issues highlight the importance of understanding interactions between water and nutrient availability.

Agriculture and forestry require more sustainable practices for plant N nutrition, that are capable of constraining environmental impact while enhancing plant fitness and productivity within the context of future climatic challenges. In this study, I undertook an *in situ* field study over 2.5 years in a low rainfall region in New Zealand, in order to investigate long-term differences in growth of ten *P. radiata* genotypes growing under organic and inorganic N supply. I hypothesised that (i) there would be genotype variation in aboveground growth responses, (ii) spatial heterogeneity in soil properties and understory vegetation would differentially influence genotype response to N source due to the variable distribution in water and nitrogen resources; and (iii) periodical resource limitations associated with inter-annual climate fluctuations would contribute to genotype strategies adopted in response to N source.

4.2 Materials and Methods

4.2.1 Site description

The experimental field site is located in the Canterbury region (Waitaha) of the South Island, New Zealand (43°28'14"S 172°20'13"E), approximately 1 km S of the Waimakariri river (Figure 4.1). This region has a temperate climate with cool, wet winters and warm, dry summers of high evaporation rates. The long term mean annual precipitation is 790 (500 - 1200) mm year^{-1} and the mean annual air temperature is 12 (6 - 17) °C between 1972 and 2015 (data obtained from NIWA Darfield station, Supplementary table B.1). The specific soil type in this trial is a Lismore Shallow and Stony Silt Loam (30-40% stone), derived from young fluvial deposits (Typic Dystrustept, USDA). This is commonly found in the Canterbury plains, characterised by being well drained, permeable, and of low water holding capacity and typically used for pastoral farming and *P. radiata* plantations. Elevations range from 110 to 115 m above mean sea level across the site (Figure 4.2b). The site was previously used for pasture followed by a first rotation *P. radiata* plantation, before a wild fire necessitated plantation re-establishment in August 2013. Logging residues from the previous rotation were windrowed and ripped along the longitudinal direction. A residual herbicide (terbuthylazine) was initially applied at registered rates to control shrub and herbaceous weeds. Weed control was no longer maintained after tree establishment, and plant communities progressively appeared. Vegetation was mainly composed of grasses, white clover (*Trifolium repens*), subterranean clover (*Trifolium subterraneum*) and annuals such as dandelions (*Taraxicum officinale*), sheep sorrel (*Rumex acetosa*), lambs ear (*Stachys sp*), wireweed (*Polygonum aviculare*), fathen (*Chenopodium album*) and mallow (*Malva sylvestris*). Less abundant species were gorse (*Ulex europaeus*) and *P. radiata* which germinated from buried seeds of the previous plantation.

4.2.2 Study design: clonal material and nitrogen treatments

The trial was planted 26 August 2013 using a planting space of 4 m (between rows) x 2 m (within rows) to give a stocking rate of 1620 cuttings ha^{-1}. The trial had a split-plot, randomised block design (Figure 4.2a), with six replicates of four N treatments applied to the main-plots: (1) NH_4NO_3, (2) L-arginine:NO_3^-, (3) L-arginine and (4) Control (no N

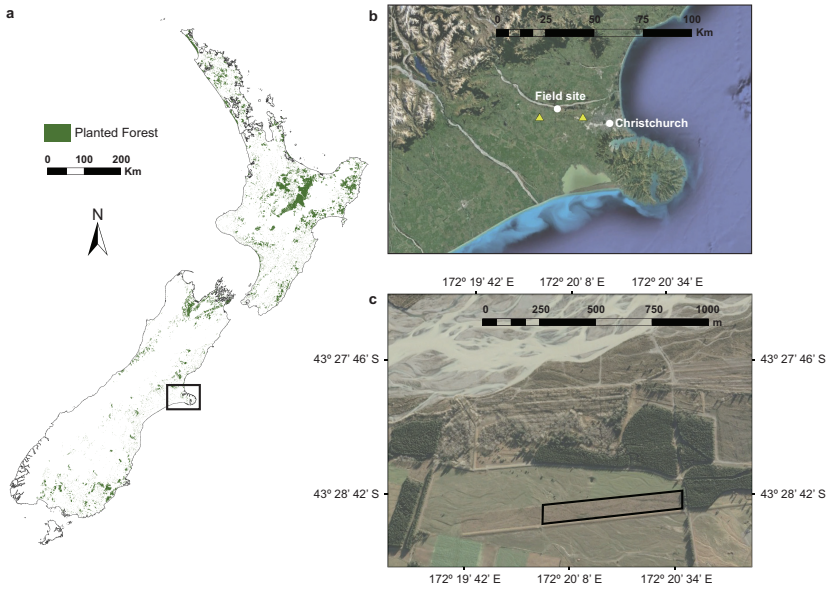

Figure 4.1: Location of the field site in the South Island of New Zealand. (a) Geographic distribution of planted exotic forest, mostly comprising *P. radiata* plantations, (b) location of the field site and weather stations (yellow triangles) in the Canterbury region, 20 km from Christchurch and (c) map of the field site. Distribution data obtained from 2016 National Exotic Forest Description report (Ministry for Primary Industries, New Zealand) and satellite maps from Google Earth.

applied). Each main-plot contained ten *P. radiata* genotypes (i.e. clones) as individual rows (subplots), i.e. each row planted with each individual genotype. Seven ramets of each genotype were randomly planted within a row, with each plot having 70 trees in total (Figure 4.2a). Space between plots was composed of at least four buffer trees (GF 19, Rangiora Nursery Limited, New Zealand). The ten genotypes (P15, P24, P28, P30, P31, P35, P37, P44, P48 and P50) were clonally propagated and provided by Forest Genetics Limited (New Zealand). The studied genotypes had a narrow pedigree (Figure 4.3) and were phenotypically selected from progeny trials based on growth rates and wood properties. N-fertilisation was carried out on each summer (December 2013 - 2015) using equimolar N concentrations and assuming 50% uptake. The N concentration of the fertiliser solutions was calculated based on the expected biomass growth requiring 2 g N g^{-1} dry weight 100. Ammonium nitrate (NH_4NO_3) was used as the inorganic form, L-arginine was used as the organic form, and the L-arginine:NO_3^- (M/M) treatment was used as the factorial combination of equimolar N concentrations of organic and inorganic N sources. For the first two years of N-fertilisation, 1 M N-solutions were used (0.5 M NH_4NO_3, 0.25

M L-arginine, and 0.13 M L-arginine and 0.5 M NO_3^- for the N equimolar combination of N forms) applied in 50 ml aliquots on each individual tree using a drench-gun. After two years of N-fertilisation (summer 2015), the N-fertiliser solutions were doubled according to tree size and the increase in plant N requirements.

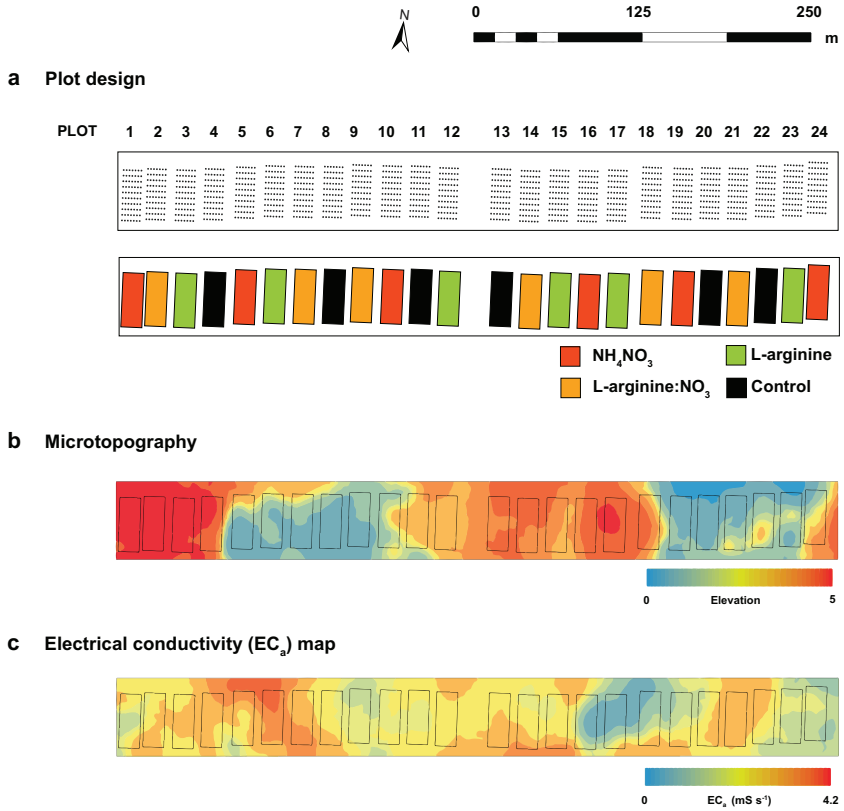

Figure 4.2: Field diagrams synthesising (a) the distribution of main-plots that contained 10 genotypes as individual rows (subplots), i.e. each row planted with each individual genotype. Seven ramets of each genotype were randomly planted within a row, with each plot having 70 trees in total randomised complete block, arranged as a split plot factor with six replicates of four N treatments applied to the main-plots, NH_4NO_3 (red), L-arginine:NO_3^- (orange), L-arginine (green) and Control (black). (b) Microtopography map at site-level interpolated from the georeferenced individual tree measures in the EM survey (c) electrical conductivity (EC_a) site-level map interpolated from individuals tree measures of apparent soil electrical conductivity of the top 160 cm of the soil (EC_{160}, mS s^{-1}).

4.2.3 Growth rate measurements and climate information

Before planting, the average dry weight biomass of the cuttings was 7.0±0.8 g DW (4.8 ± 0.3 g shoot DW, 2.4 ± 0.6 g roots DW), with no significant genotype variation in dry

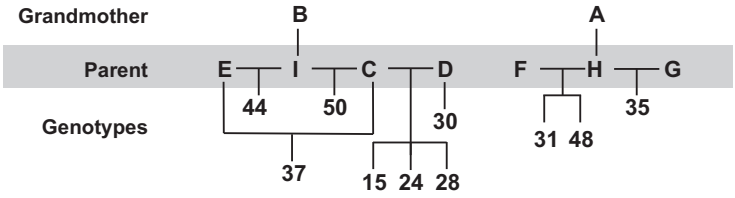

Figure 4.3: Pedigree of the *Pinus radiata* genotypes used in the experiment. Capital letter represent the coded series of the ancestors. Grandmothers and parental ancestry were selected by *Radiata Pine Breeding Company Ltd* and the resulting progeny was selected by *Forest Genetics Ltd.*

biomass. Nitrogen additions were applied in December 2013 at the same time of the first tree root collar diameter (±0.1 mm) and tree height (±0.1 cm) measurement of individual trees. Measurements were repeated in October 2014, May 2015, December 2015 and June 2016. Estimates of tree volume were calculated assuming a conic stem shape, following the equation $V_{tree} = \frac{1}{3}\ \pi r^2 h$, in which r and h correspond to the radius of the root collar and height of the stem, respectively. Volume increments ($\Delta V_{(t,t+1)} = V_{t+1} - V_t$) were used to describe growth over time, t and $t+1$ describe initial and final tree volume after a time period, respectively. The cumulative incremental volume was calculated for the four periods between: December 2013 and October 2014 ($T_1 - T_0$), December 2013 and May 2015 ($T_2 - T_0$), December 2013 and December 2015 ($T_3 - T_0$) December 2013 and June 2016 ($T_4 - T_0$). In October 2014, the percentage of understory vegetation (percentage cover) and the proportion of annual, clover and grass plant communities were visually determined within a 1 m diameter of each tree. The climatic data for the years of the study was obtained from the closest NIWA (National Institute of Water and Atmospherics Research) weather station 15 km east from the site (agent 41230, 43°29'22"S 172°31'39"E) (Figure 4.1b). The average of monthly cumulative rainfall (mm) and mean daily air temperature (°C) on each individual time period (T_n) were used to explain differences in volume increment (see subsection 4.2.5). The historic record of climatic data between 1972 and 2015 was obtained from another NIWA station 16 km west from the site (agent 4836, 43°29'35"S 172°08'13"E) (Figure 4.1b, Table B.1). The relative humidity (%) was calculated using mean vapour pressure (hPa) and the saturated vapour pressure at the given temperature.

4.2.4 Electromagnetic survey and apparent electrical conductivity map

In September 2014, a soil conductivity meter Dualem-1 (DUALEM, Milton, ON, Canada) with simultaneous collection of georeference was used to measure apparent soil electrical conductivity of the top 160 cm of the soil (EC_a, mS s^{-1}). A series of off-sets were collected prior to, and again at the completion of measurements to calibrate for environmental changes during the time required to collect measures. Using a systematic approach, rows 1 to 10 were assessed in plots 1 through 24, using a zig-zag pattern. The instrument was laid on the ground directly beside each tree and the electrical conductivity response was collected by manual trigger. The orientation of the instrument was in the WE and EW directions (following rip lines) depending on the row assessed. The instrument was randomly placed on either the left or the right hand side of the tree depending on the operator and muscle fatigue. Approximately half way through the plot assessments, five soil moisture samples were collected from the first plot in the western side and the instrument batteries were replaced. Data was then adjusted to account for the off-sets and other changes as per normal operational recommendations. Interpolated EC_a and elevation maps (Figure 4.2b,c) were made by kriging in ArcMap (Environmental Systems Research Institute, Redlands, CA) and its extension of Geostatistical Analyst.

4.2.5 Statistical analyses

Data were analysed using R Studio (Version 0.99.902) (R Core Team, 2012). The effects of plot, N source and genotype in volume increment over the length of the experiment were tested using linear models followed by one-way analysis of variance (ANOVA) tests. A two-way ANOVA was conducted to test the significance of genotype, N source and the interaction of both in volume growth response. Given the significant interaction in the the previous test, one-way ANOVA tests were conducted to analyse the effect of N source in the volume increment for individual genotypes. Tukey's Honest Significant Difference (HSD) test was used to compare means ($p < 0.05$) when significant effects were found.

To test the genetic and environmental factors influencing overall volume increment between December 2013 and June 2016, a linear mixed model was conducted including as predictor variables: genotype, N source, EC_a and percentage understory vegetation (and all their interactions) as fixed effects, and considered Plot and Row as random effects. Plots

were nested in N source and Rows nested to genotypes. To model the effect of climatic factors in the volume increment over four time periods between December 2013 and June 2016 (see section 4.2.3) linear-mixed models were also computed. Here predictor variables were: cumulative rainfall (mm), air daily mean temperatures (°C) and the interaction between both, averaged in each of the time periods, as fixed effects. Random effects were genotype, N source and the interactions between both. Statistical significance of fixed predictors was assessed using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom in the package lmerTest (Kuznetsova *et al.*, 2015) and of random effects using likelihood ratio tests. In both linear mixed models, volume increments were normalised with logarithmic transformation in order to meet the assumptions of homoscedasticity and normality. In all models, volume increments were normalised with logarithmic transformation in order to meet the assumptions of homoscedasticity and normality. Following analysis, response variables and coefficients were back-transformed for the interpretation.

4.3 Results

4.3.1 Genotype-specific growth response to nitrogen source

The study revealed great variability in growth increment throughout the site (Figure 4.4) with significant differences in volume growth across plots ($F_{(23,1560)} = 10.15$, $p < 0.001$). Maximum growth was observed in plots 6, while the lowest growth was observed in plots 11 and 12. Variation in tree growth was significantly influenced by tree genotype ($F_{(9,230)} = 4.09$, $p < 0.001$)(Table 4.1), while the effect of N treatment was not a main source of variation. Full-sib genotypes 48 and 31 had the greatest growth increment, with genotype 15 possessing the slowest average growth (over 15% change than genotype 48).

Furthermore, the significant effect of the interaction between N source and tree genotype on the relative tree growth ($F_{(27,230)} = 2.94$, $p < 0.001$) indicated the presence of genotype-specific growth response to N source. Genotypes 30, 48 and 50 showed a significant response to N source, while the remaining seven (i.e. 15, 24, 28, 31, 35, 37 and 44) presented a neutral response or non-significant response to N treatment. One-way ANOVA tests showed that genotypes 30 and 50 were organic N-responsive genotypes,

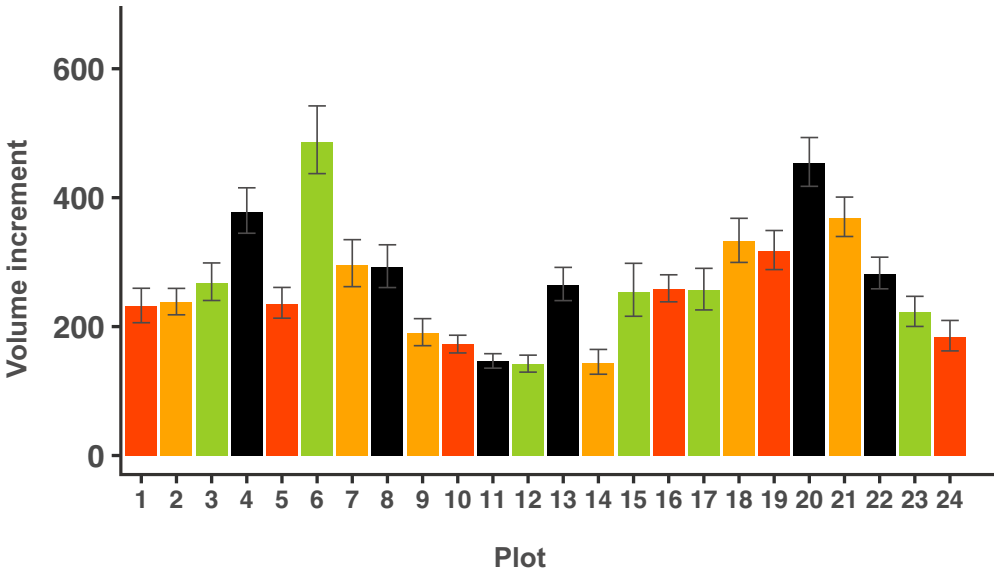

Figure 4.4: Volume increment (cm^3) across plots between December 2013 and June 2016. Colouring of bars show the six replicates of four N treatments applied to the main-plots, NH_4NO_3 (red), L-arginine:NO_3^- (orange), L-arginine (green) and control (black). Data are presented as mean ± SD, after back-transformation from logarithm scale.

Table 4.1: Effect of tree genotype in the mean volume increment (ΔV, cm^3) between December 2013 and June 2016 in *P. radiata*. Results were averaged by genotype without considering the N source effect. Mean ± SD with different letters are significantly different (Tukey *post hoc* pairwise comparison, $p < 0.05$). Volume values obtained after back-transformation from logarithm scale. *N* number of individual replicates.

Tree genotype	N	$\Delta V \pm SD$
15	24	$172.21 \pm 2.60^{\ e}$
24	24	$235.74 \pm 2.27^{\ cde}$
28	24	$306.11 \pm 2.23^{\ bc}$
30	24	$218.49 \pm 2.71^{\ de}$
31	24	$343.56 \pm 2.36^{\ ab}$
35	24	$258.54 \pm 2.29^{\ bcd}$
37	24	$207.36 \pm 2.37^{\ de}$
44	24	$203.38 \pm 2.43^{\ de}$
48	24	$430.92 \pm 2.27^{\ a}$
50	24	$250.89 \pm 2.48^{\ cd}$

with significantly greater performance in response to L-arginine than to NH_4NO_3, while genotype 48 responded greater to the factorial combination of organic and inorganic N forms (L-arginine:NO_3^-), than to fertilisation with L-arginine alone. Genotypes showed apparent heritability of growth rate-related traits and/or response to N source, especially in those with full-shared parental identity (see pedigree in Figure 4.3). For instance, full-

sib 31 and 48 had the greatest aboveground growth (Table 4.1), despite showing different response to N source, while full-sib genotypes 15, 28 and 24 similarly showed a non-significant response to N source (neutral response) but exhibited significantly different growth increment.

Table 4.2: Genotype-specific response to the different N sources, NH_4NO_3, L-arginine:NO_3^-, L-arginine and control; based on the significance of individual one-way ANOVAs in response to N source. When significant, the test followed a Tukey *post hoc* pairwise comparison between the volume increment means in each of the N treatments. Mean ± SD with different letters are significantly different (Tukey *post hoc* pairwise comparison, $p < 0.05$). Volume values obtained after back-transformation from logarithm scale.

	Volume increment (cm^3)				
	NH_4NO_3	**L-arginine:NO_3^-**	**L-arginine**	**Control**	***p*-value**
Genotype 15	158.98±2.35	149.28±2.77	154.32±3.24	234.44±2.09	0.141
Genotype 24	183.10±2.17	228.16±2.71	285.86±2.23	258.29±1.89	0.100
Genotype 28	280.36±2.00	352.55±2.37	252.32±2.48	350.21±2.05	0.167
Genotype 30	138.01 ± 2.65^{b}	227.33 ± 2.21^{ab}	280.16 ± 3.16^{a}	246.18 ± 2.53^{a}	**0.009**
Genotype 31	357.51±2.02	250.63±1.96	379.71±2.94	405.33±2.34	0.052
Genotype 35	264.09±2.06	262.75±2.23	195.95±2.71	325.92±2.07	0.060
Genotype 37	265.69±2.16	188.02±2.42	161.66±2.24	228.61±2.54	0.054
Genotype 44	200.84±2.03	209.71±2.80	201.96±2.58	201.14±2.40	0.996
Genotype 48	393.26 ± 1.84^{ab}	611.91 ± 2.35^{a}	341.55 ± 2.71^{b}	424.29 ± 2.02^{ab}	**0.013**
Genotype 50	156.36 ± 2.57^{c}	222.28 ± 2.15^{bc}	436.04 ± 2.25^{a}	262.03 ± 2.23^{b}	**< 0.001**

4.3.2 Effect of the microsite variability on the genotype responses to nitrogen source

The estimate of soil apparent electrical conductivity (EC_a) in proximity to each individual tree had a range between 0.7 and 3 mS s^{-1} when measured early in spring 2014 (October). The significant differences in electrical conductivity across plots ($F_{(23,1560)} = 39.06$, $p < 0.001$) indicated that topography and soil properties were heterogeneous throughout the site, both within and between experimental plots ($p<0.001$) (Figure 4.2b - 4.2c). At the same time, the understory vegetation was dominated by seasonal plants (Figure 4.5), mostly grasses and clovers, with approximately 20% cover of each. The extent of vegetation cover (annual and seasonal plant species) was significantly increased by the effect of N source ($F_{(3,1576)} = 6.29$, $p < 0.001$) as was annual plant species ($F_{(3,1580)} = 5.17$, $p =$

0.001) and grass cover ($F_{(3,1580)}$= 5.16, p = 0.002), but N source did not influence the cover of clover ($F_{(3,1580)}$ = 1.57, p = 0.198) (Figure 4.5).

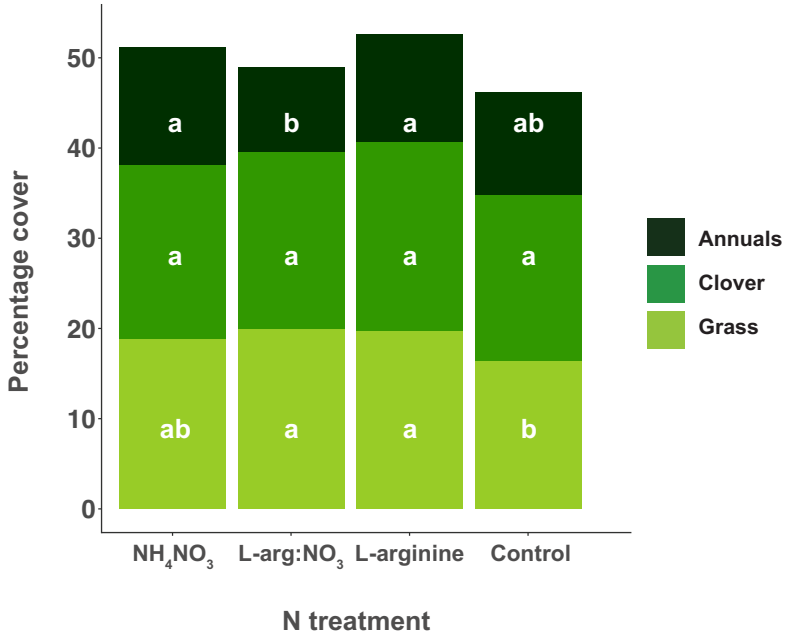

Figure 4.5: Differences in the percentage of understory vegetation composed by annual plants (*Taraxicum officinale*, *Rumex acetosa*, *Stachys sp*, *Polygonum aviculare*, *Chenopodium album*, *Malva sylvestris* and *Ulex europaeus*), clovers (*Trifolium repens*, *Trifolium subterraneum*) and grasses, in response to N source. The assessment was undertaken in September 2014 (spring). Letters in bars within the same plant type indicate significant differences across N treatments using Tukey *post hoc* pairwise comparison ($p < 0.05$).

An analysis of variance was conducted to test whether the genotype-specific growth response to N source was further influenced by soil descriptors and the understory vegetation (Table 4.3). The analysis showed that differences in incremental volume were significantly influenced by the interactive effect of tree genotype, N source, EC_a and the cover of understory vegetation (annual, clover and grass plant species).

Table 4.3: Summary of the analysis of variance (4-way ANOVA Type III) conducted to test the effect of predictor variables genotype, N source, apparent electrical conductivity (EC_a), understory vegetation cover (Cover) - including annual, clover and grass plant types - and the interaction of them in the increment of tree volume between December 2013 and June 2016 in *P. radiata*. Only interactions with significant p-values (< 0.05) are shown.

Predictors	**F-value**	***p*-value**
Genotype	$F_{(9,1563.5)} = 0.43$	0.920
N source	$F_{(3,1518.1)} = 2.33$	0.072
EC_a	$F_{(1,1577.7)} = 11.85$	$<$ **0.001**
Cover	$F_{(1,1573.0)} = 0.11$	0.741
Gen. x N	$F_{(27,1563.4)} = 2.17$	$<$ **0.001**
N x EC_a	$F_{(3,1574.7)} = 2.90$	**0.034**
Gen. x N x EC_a	$F_{(27,1563.0)} = 2.57$	$<$ **0.001**
Gen. x N x Cover	$F_{(27,1562.6)} = 2.23$	$<$ **0.001**
Gen. x N x Cover x EC_a	$F_{(27,1562.1)} = 2.76$	$<$ **0.001**

4.3.3 Effect of climatic factors on the incremental growth response to nitrogen source

Climatic conditions differed seasonally between 2013 and 2016, with a wet cold summer (T_1) preceding a year with low rainfall (T_2, T_3) (Figure 4.6). Monthly cumulative rainfall in autumn 2014 was higher than the average of the last 40 years (1972 - 2015)(Table B.1). Contrasting conditions were also recorded for minimum temperature, in which the first quarter of years 2015-2016 had higher average temperatures than in 2014. The contrasting inter-annual changes in precipitation and minimum temperature were accompanied by a consistent increase in the monthly maximum temperatures in autumn (2014-2016) over the years of study. Average relative humidity changed in a similar manner to the rainfall, but was within the normal historical ranges (Table B.1).

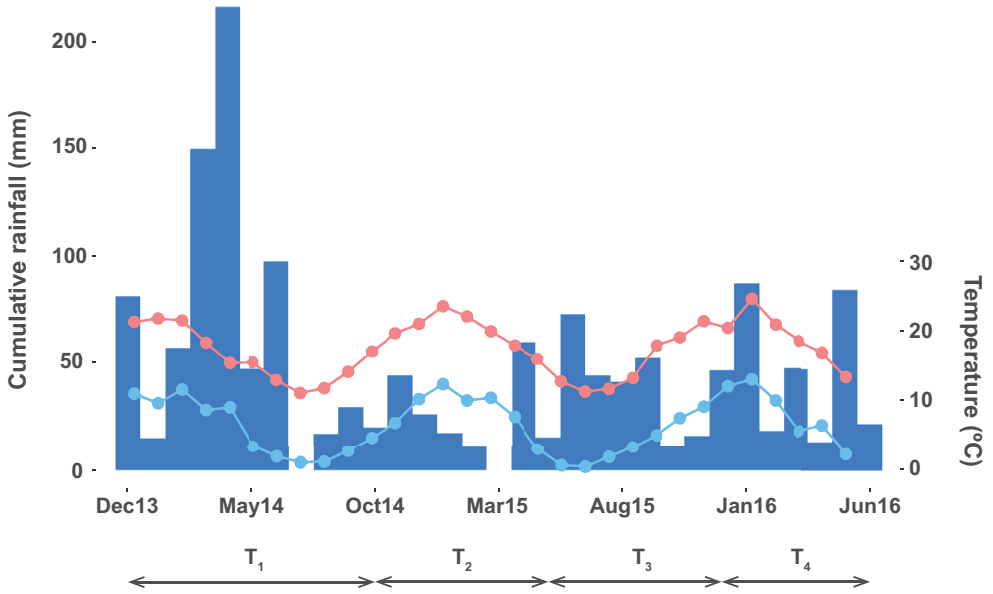

Figure 4.6: Monthly cumulative rainfall (mm), daily mean maximum (red dots) and minimum temperatures (light blue dots)(°C) between December 2013 and June 2016 obtained from the closest NIWA (National Institute of Water and Atmospherics Research) weather station, 16 km from the site (agent 41230, 43°48'S 172°52'E). Arrows define the four time periods (T_1, T_2, T_3, T_4) in which tree measures were undertaken (December 2013, October 2014, May 2015, December 2015 and June 2016).

As a result of these climate fluctuations, fertilisation with different N source influenced the incremental volume growth over the time periods between December 2013 and June 2016. The effect of N source significantly influenced the patterns of cumulative in-

cremental growth in tree volume, although this effect varied depending on the time period (Figure 4.7). Growth increased consistently over time, and approximately 50% of the total growth occurred between December 2015 and June 2016 (T_4). After the tree establishment until October 2014 (T_1), the increment in tree volume was greater ($p = 0.046$) for trees growing under L-arginine than in inorganic N (T_1, $p = 0.050$). After October 2014 and until December 2015 trees reached a similar volume, as shown by the non-significant effect of N source (T_2, $p = 0.078$; T_3, $p = 0.173$). From December 2015 towards the end of the experiment, significant differences were found in cumulative growth in response to N source (T_4, $p = 0.004$), in which untreated trees showed greater incremental growth than those with applied inorganic N. Trees fertilised with the combined treatment of organic and inorganic N forms did not show significant differences in the cumulative incremental volume from trees supplied with either N form alone.

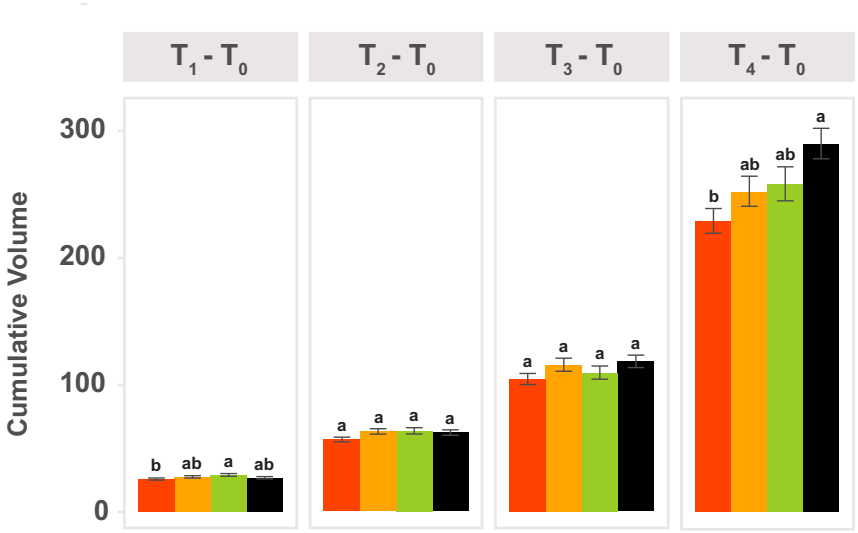

Figure 4.7: Cumulative incremental volume (cm^3) between December 2013 (T_0) and October 2014 (T_1 - T_0), May 2015 (T_2 - T_0), December 2015 (T_3 - T_0) and June 2016 (T_4 - T_0). Colour of bars indicate the N treatment supplied, NH_4NO_3 (red), L-arginine:NO_3^- (orange), L-arginine (green) and control (black). Letters above bars indicate significant differences between N sources using Tukey *post hoc* pairwise comparison following a one-way ANOVA ($p < 0.05$).

The relationship between volume increment for each individual time period (V_{t+1} - V_t) and climatic variables was influenced by N source and tree genotype. Linear mixed modelling (Table 4.4) indicated that climatic descriptors were useful predictors of growth and the interaction of monthly cumulative rainfall and air daily mean temperatures was better than either alone. Tree volume was negatively associated with increasing tem-

perature (drought) ($\chi^2_{(2)}$=2351, $p < 0.001$). Every degree (°C) increase in the mean temperature reduced volume by a 70% change. The increase in 1 mm cumulative rainfall ($\chi^2_{(2)} = 2120.4$, $p < 0.001$), decreased volume by 34.7% change and the interaction of both ($\chi^2_{(1)}$=2118.9, $p < 0.001$) had a lesser but significant effect, increasing volume by 3.6% change when temperature and rainfall increased together. In addition, the significant effect of the interaction between genotype and N source as random effects (Table 4.4), showed that the effect of climate on growth varied depending on the genotype response to N source.

Table 4.4: Climatic factors (cumulative rainfall and air daily mean temperature averages) predicting the volume increment within the four time periods between December 2013 and October 2014 (T_1 - T_0), October 2014 and May 2015 (T_2 - T_1), May 2015 and December 2015 (T_3 - T_2) and December 2015 and June 2016 (T_4 - T_3). Statistical significance of fixed predictors was assessed using Type III ANOVA with Satterthwaite's approximation of denominator degrees of freedom. Genotype, N source and the interaction between genotype and N source were used as random effects in the model and the significance was assessed with likelihood ratio tests using ML estimation. Bold *p*-values denote statistically significant differences (< 0.05).

Fixed effects	**F-value**	***p*-value**
Rainfall	$F_{(1,6157.1)} = 762.84$	$<$ **0.001**
Temperature	$F_{(1,6157.3)} = 567.27$	$<$ **0.001**
Rain. x Temp.	$F_{(1,6157.1)} = 784.94$	$<$ **0.001**
Random effects	χ^2	***p*-value**
Genotype	$\chi^2_{(1)} = 4.66$	**0.030**
N source	$\chi^2_{(1)} = 1.15$	0.284
Gen. x N	$\chi^2_{(1)} = 37.05$	$<$ **0.001**

4.4 Discussion

The study has revealed a strong genetic variation in *P. radiata* growth capacity along with a variable degree of phenotypic plasticity in growth responses to environmental factors. These results highlight the strong microsite effect on the variability in soil properties and the heterogeneous spatial distribution of resources at field-scale, which can strongly impact genotype response to available N sources. This reveals the potential underestimation of effects when averaging experimental plots in field studies with no information regarding the soil properties of the site. Previous studies of conifers have reported greater growth with NH_4^+ than with NO_3^- treatment (Van Den Driessche, 1971), although others have shown enhanced growth in early-successional ecosystems with high soil nitrate content (Parfitt

et al., 2003). This suggests that plant species tend to acclimate to the available N source (Kronzucker *et al.*, 1997). Strong interactions between genotype and nutrient availability have been observed among families of *P. taeda* (Crawford *et al.*, 1991) and *P. radiata* (Hawkins *et al.*, 2010), sometimes leading to differences in leaf nutrient content within sites (Burdon, 1976). There are very few studies that have reported variation in growth rates of conifers growing in/with organic and inorganic N forms, and generally the results were inconclusive (Gruffman *et al.*, 2012; Wilson *et al.*, 2013). Nevertheless, fertilisation with organic N is generally associated with differences in biomass allocation, mainly characterised by greater root allocation (root:shoot) compared to inorganic fertilisers (NO_3^-) (Cambui *et al.*, 2011; Franklin *et al.*, 2017). Numerous models have proposed that biomass allocation is regulated by the internal N status, and nutrient limitations on plant growth lead to an increase in allocation to roots and reduction of shoot growth (Thornley, 1972). While measures of biomass distribution were not possible in this non-destructive study, it is likely that limitations in soil N availability led to differences in biomass allocation.

Genotypes with an overall greater volume were also those that responded strongly to fertilisation with organic N or the combination of N forms (i.e. genotypes 48, 30 and 50). Preference for a N source can be driven by several factors including an increased affinity of specific transport systems (e.g. amino acid transporters - Persson & Näsholm, 2001) or enhanced utilisation and hence growth in response to the form supplied (Britto & Kronzucker, 2013). There are two possible scenarios that could explain the apparent genotype preferences for N source and its linkage to growth capacity. The first scenario suggests that the genotypic variation in the capacity for acquisition of different N sources might leads to changes in the overall growth (Harrison *et al.*, 2007; Boczulak *et al.*, 2014). A number of studies have also shown some conifers may take up organic and inorganic N sources at comparable rates (Miller & Cramer, 2004; Öhlund & Näsholm, 2004). However, Brackin *et al.* (2015) recently attributed the low N efficiencies of inorganic forms in crop systems, such as sugar cane, and the N loss to the environment, to the the capacity of the root to absorb inorganic N being exceeded at certain levels of application, in contrast to organic N that more closely matched the capacity of the root. The second scenario suggests that genotypes with an overall greater performance might be less sensitive to changes in N availability, because of the greater efficiency of N use (Bongarten *et al.*, 1987; Garcia

Villacorta *et al.*, 2015). In fact, recent studies have reported that treatments with organic N forms may contribute more efficiently than inorganic forms to a plant N budget, even at lower concentrations, given the lower carbon cost of assimilation (Franklin *et al.*, 2017). In addition to the variation in genotype-specific strategies of uptake and use of nutrients, fertilisation with different N source may lead to differences in N availability through competitive effects with weeds, thereby influencing tree growth. The greater grass cover in seasonal grasses observed in organic N treatments (with and without NO_3^-) compared to untreated trees, indicate the potential for greater N retention in the rhizosphere of trees fertilised with L-arginine compared to inorganic N sources.

Heterogeneity in soil properties influenced the way N source impacted plant growth. This likely resulted in changes in the availability of N sources that constrained root absorption. The spatial variability in soil properties is supported by the lower EC_a values obtained in the EM survey, which have been associated with a generally low water availability and the absence of top-soil organic matter (Sudduth *et al.*, 2005). In addition, the topographic differences were characterised by an elevation range of 5 m, lower old river channels, may be more sheltered and closer to the water table. Conversely, plants at the higher elevations of the site might be impacted by exposure to stronger winds and associated atmospheric water stress. Water retention and availability was possibly a key determinant of plant N availability, by differently influencing the retention of the N chemical forms of distinct molecular structures (Owen & Jones, 2001). Firstly, water is the medium via which nutrient movement and uptake occurs. In this respect, nutrient mobility is important - Öhlund & Näsholm (2002) reported that L-arginine is subject to strong soil immobilisation, due to the negative charge of soil particles, in contrast to the high mobility of NO_3^-. Secondly, soil water content also impacts soil biotic processes and alters the metabolic activity of soil decomposers in soil microbial communities (Manzoni *et al.*, 2012) resulting in changes in heterotrophic respiration and nutrient mineralisation. Therefore, soil moisture content, driven by soil water holding capacity and the variability in rainfall, might contribute to differences in tree growth through a complex mix of indirect effects involving rates of nutrient supply and interconversion between N forms, but tempered by plant root-associated microbial interactions (i.e. competitive, symbiotic) influencing nutrient availability and the absorption capacity of the plant (Harrison *et al.*,

2008; Näsholm *et al.*, 2013).

In addition to the influence of G x E interactions on plant growth, seasonal changes in climatic conditions, driven by variable patterns of rainfall and daily mean temperature, also influenced the extent to which genotypes responded to N source over time. This agrees with previous studies that report a certain degree of phenotype flexibility in *P. radiata* induced by unfavourable climate conditions (Burdon *et al.*, 2001). The response of cumulative incremental volume over time shows that towards the end of the experiment, there is an increase in the overall growth rates as well as a maximisation of differences between N source. This suggests the effect of climate and associated factor were critical for the first two years after tree establishment. It is interesting that unfertilised trees had a cumulative incremental volume that was not significantly different to the fertilised trees until December 2015, then from this time until June 2016 displayed greater growth than trees under L-arginine and L-arginine:NO_3^- treatment. A possible explanation for the greater delayed growth in untreated compared to N-fertilised trees might be the overextension of resources by N-fertilised trees under favourable environmental conditions that became unsustainable under less favourable conditions. Leaf traits were not measured, but fertilisation generally promotes greater investment in needles (Fife & Nambiar, 1997). As a result, for a given transpiration rate, fertilisation could potentially increase the total evapotranspiration of the plant, which could overly stress the plant under dry conditions (Irvine *et al.*, 1998; Miyazawa & Lechowicz, 2004). As explained above, the combination of porous soil and the presence of periods with very low precipitation, likely accentuated genotype x drought responses in growth. Research on genetic variation in foliar $\delta^{13}C$ indicates that there may be genotype-specific responses under water stress conditions that determine phenotype adaptability to water tolerance (e.g. Cregg & Zhang, 2001; Prasolova *et al.*, 2003). Lim *et al.* (2015) recently proposed that changes in inter-annual precipitation may help explain the reported large variability in growth responses to fertilisation of pine stands. This agrees with my results that indicate that the effects of rainfall and temperature significantly impacted growth, depending on genotype and the N source. This could imply that different genetic strategies influencing water efficiency might determine tree response to environmental N changes.

4.5 Conclusions

Three patterns emerged from this field experiment. First, the results align with prior findings that growth in temperate conifers is strongly influenced by the genotype-by-environment interaction, with variation of phenotypic expression of different *P. radiata* genotypes to N-fertilisation despite the relatively narrow pedigree of the studied trees. Second, *P. radiata* showed genotype-specific responses to fertilisation with different N source (NH_4NO_3, L-arginine:NO_3^-, L-arginine). Third, the interactive effect of the spatial heterogentity of soil properties, spatial distribution of understory vegetation and N source determined genotype performance and the susceptibility of trees to climatological fluctuations. The results suggest that the forestry sector requires a better understanding of the flexibility and adaptability of genotypes beyond the capacity for growth and towards efficient strategies of N use. This may have important implications for the establishment of criteria in breeding programmes considering the ecological impact of climate change on tree genetics. N-fertilisation in forest plantations could be optimised by using site-specific N regimes and N source that adjust to the soil characteristics of the site and to climate in order to increase sustainability - maximise fertiliser response and minimise N leaching - to improve stand productivity beyond the establishment phase. In conclusion, silvicultural practices must acquire a more complex appreciation that utilises tree selection and plantation management operations that consider both biotic and abiotic factors as they influence site quality.

Chapter 5

Genotypic variation in *P. radiata* responses to nitrogen source are related to changes in the root microbiome

5.1 Introduction

Recent research has highlighted the important contribution that belowground communities, in particular bacteria and fungi associated with plant roots, make to aboveground productivity and other ecosystem functions. These root-associated communities play a major role in plant health and adaptation to environmental stress (Panke-Buisse *et al.*, 2015). Traditionally, such benefits were associated with particular taxa (i.e. symbiotic mutualists), including mycorrhizal fungi. Mycorrhizas are symbiotic interactions between specialised fungi and plants (Brundrett, 2002), and their main role is to trade resources, by exchanging photosynthates for nutrients, which the fungal partner provides by way of an extensive and enzymatically dynamic hyphal system (Schimel & Bennett, 2004; Smith & Read, 2008). However, it is now recognised that to understand above-belowground linkages, the entire rhizosphere community, and interactions ranging from mutualistic to competitive, must be considered (Wardle, 2006). Root-associated microbes not only can act synergistically to directly impact plant development, but they also mediate soil biogeochemical cycles (reviewed by Lambers *et al.*, 2009). Via these direct and indirect mechanisms, organisms in the rhizosphere play key roles in determining plant productivity and community composition (Bever *et al.*, 2010; Lau & Lennon, 2012). The taxa present in the rhizosphere of plant species (and their diverse genotypes) can now be efficiently characterised with next-generation sequencing approaches (e.g. Bulgarelli *et al.*, 2015; Lamit *et al.*, 2016). However, the underlying mechanisms that determine the com-

position of these root-associated communities remain unclear. Of particular importance are the relative roles of biotic and abiotic factors, specifically genotypic variation in plant characteristics, soil conditions, and their interaction.

Both soil physicochemical properties and plant identity have been shown to contribute to the assembly of rhizosphere communities (Berg & Smalla, 2009). It has been proposed that edaphic factors are the primary filters determining the biota that roots encounter (Fierer & Jackson, 2006). Plant traits then determine which taxa can establish in the rhizosphere (Bulgarelli *et al.*, 2013), and these traits can vary both among and within species. Variation among plant species (Haichar *et al.*, 2008) and genotypes (Micallef *et al.*, 2009) in quantity and type of rhizodeposits (organic compounds released from plant roots) is associated with differences in soil communities. Rhizodeposition patterns can also change with plant development, nutritional status, and environmental stress (Bever *et al.*, 2012; Chaparro *et al.*, 2014), potentially inducing shifts in the composition or diversity of root-associated communities. Plants roots can also release or absorb ions that induce changes in the rhizosphere soil characteristics (i.e. oxygen, pH), which can in turn influence soil processes, including nitrogen cycling, and this capacity varies among and within species (Liu *et al.*, 2004; Blossfeld *et al.*, 2011). In addition, intraspecific variation in aboveground plant traits, such as leaf secondary compounds, has been shown to significantly impact soil microbial communities and soil processes (reviewed by Schweitzer *et al.*, 2008a). Finally, plants exhibit intraspecific variation in their responses to abiotic conditions (Osorio & Pereira, 1994; Rengel & Marschner, 2005), which may in turn impact rhizosphere communities.

An important way in which plant genotypes vary is in their ability to access (root morphology) and acquire (root transporters) soil resources (reviewed by Rengel & Marschner, 2005), yet the extent of intraspecific variation in ability to take up and assimilate different nitrogen (N) forms is not well understood. Nitrogen in soil ranges from simple inorganic forms (NH_4^+ and NO_3^-) to organic forms (amino acids, peptides and proteins), and microbially-mediated processes limit the availability of these molecules (Reich *et al.*, 1997; Schimel & Bennett, 2004). Plants can only take up a portion of the N present in the soil solution (NH_4^+, NO_3^- and free amino acids). While all major groups of mycorrhizal and non-mycorrhizal species tested thus far have exhibited the capacity to take up amino

acids, the relative contributions of inorganic and organic N forms to plant N budgets remains unclear (Näsholm *et al.*, 2009). It has been suggested that a plant's capacity to establish symbiotic relationships with mycorrhizal fungi may increase its ability to access a broader range of N forms (Read & Perez-Moreno, 2003). The ability of plants to take up amino acids may be a competitive advantage, as amino acids serve as C and N sources for soil microbes which can compete with plants for this resource (Hodge *et al.*, 2000; Kuzyakov & Xu, 2013). Fertilisation with organic N can result in changes in plant development, including greater rates of mycorrhizal colonisation (Turnbull *et al.*, 1995) and differences in biomass distribution (Cambui *et al.*, 2011) when compared to inorganic N supply. However, the degree to which these responses to N form vary among plant genotypes, and whether such variation translates to variation in root-associated communities, has not been established. Understanding such relationships is essential, because differences in the quality and quantity of soil resources can change the nature of interactions in the rhizosphere and alter plant-soil feedbacks (Bell *et al.*, 2014). In addition, root associated microbial communities may be influenced directly by additions of inorganic versus organic N due to the presence of labile carbon in the organic N source (Jones *et al.*, 2005a), potentially altering nutrient availability in the rhizosphere.

There is increasing evidence that variation in traits within a plant species can drive differences in soil chemistry and soil microbial communities. However, little is known about how intraspecific variation in plant responses to abiotic conditions affects the diversity and composition of the rhizosphere, either directly (by changing the nature of plant-microbe interactions) or indirectly (by altering soil properties in ways that favour particular microbial taxa). Here I present results from a field experiment investigating how the root microbiomes of two full-sib *P. radiata* genotypes with distinct physiological responses to additions of organic versus inorganic N (Chapter 2, Chapter 3), were altered by two years of fertilisation with different N forms. Rhizosphere bacterial and fungal communities were characterised, as well as root fungal communities, of trees receiving yearly additions of NH_4NO_3 or L-arginine, and control trees. Plant traits (height, needle nutrient content, water use efficiency, and ectomycorrhizal colonisation rate) and soil nutrient content were measured for the same trees to link variation in tree responses to variation in rhizosphere and root communities. With this approach I was able to determine whether tree genotypic

variation in response to nitrogen form is reflected in the rhizosphere, thus extending the understanding of genotype by environment interactions to host-associated communities.

5.2 Materials and Methods

5.2.1 Experimental design

The root microbiomes of 2-year-old *P. radiata* trees were characterised. Samples were collected from a field trial designed to assess genotype variation in growth rate in response to organic and inorganic N sources, and to compare these N treatments to untreated trees. The trial was located in the Canterbury region (Waitaha) of the South Island, New Zealand (43°28'S 172°20'E) on a Lismore Shallow and Stony Silt Loam (30-40% stone) soil type from fluvial deposits from the Waimakariri River [classified as Pallic Orthic Brown Soil (NZ) and Typic Dystrustept (USDA)] (Figure C.1a). The trial was established in August 2013. The site was burned approximately 12 months before planting, and logging residues from the previous rotation were windrowed and ripped along the length of the experimental plot. The split-plot design (Figure C.1b) consisted of three main-plots corresponding to the three N treatments (control, inorganic N or organic N). Seventy trees were planted in each of the main plots with ten genotypes of *P. radiata* randomly distributed in rows (sub-plots). Genotypes planted were clonally propagated and phenotypically selected in progeny trials based on growth rates and wood properties (Forest Genetics Limited, New Zealand).

The organic and inorganic N treatments consisted of the addition of 1 M N solutions as either L-arginine (0.25 M) or NH_4NO_3 (0.5 M), respectively. This N fertilization level was determined by estimating the expected annual growth (2% N g^{-1} DW) and assuming 50% root uptake. Between 2013 and 2015, trees were fertilised individually once per year during summer (December - February) by applying 50-ml aliquots with a fixed volumetric drench-gun to homogeneously distribute nutrients around the tree base. No other macronutrients were provided. The last N treatment was applied two weeks before the collection of the soil and root samples.

The root-associated communities of four replicate trees from each of two full-sib genotypes (31 and 48) in each of the main-plots were assessed in this study. These

genotypes were selected because they demonstrated intraspecific variation in aboveground growth in response to organic and inorganic N forms in a previous greenhouse study (Chapter 2). As soil and climate parameters were likely to vary across the trial, all measured trees were from the western end of each block. There was a drought at the time of sample collection (13 February 2015). The cummulative rainfall between December 2014 - February 2015 (summer season) was 54.6 mm, much lower than the average historic record of 182.5 mm ± 63.7 (mean ± SD) for this period over the years 1973 - 2014 (data obtained from New Zealand National Institute of Water and Atmospheric Research station at 43°29'S 172°08'E).

There was no weed control after tree establishment. Weed cover was assessed in September 2014 and was mainly composed of grasses, clovers (*Trifolium repens* and *Trifolium subterraneum*), annuals (dandelions (*Taraxicum officinale*), sheep sorrel (*Rumex acetosa*), lambs ear (*Stachys sp*), wireweed (*Polygonum aviculare*), fathen (*Chenopodium album*) and mallow (*Malva sylvestris*). Gorse (*Ulex europaeus*) was present, but less abundant.

5.2.2 Sample collection and tree growth measurements

Needle, root and soil samples were collected from four trees of each genotype (31 and 48) from each of the three N treatments (control, inorganic N, and organic N). Samples were collected in late summer (13 February 2015). For each individual tree, approximately 200 g of soil was collected by pooling four cores (15 cm depth and 5 cm diameter) taken 20 cm from the base of the tree. Soil samples were stored in sealed plastic bags and placed on dry ice during transport to the laboratory, then stored at -20°C. Tree root collar diameter (±0.1 mm) and height (±0.1 cm) were measured when soil cores were taken. A 2-3 g sample of new-growth needles was also collected from each tree, stored in sealed plastic bags and placed on dry ice during transport. Needle fresh weight was determined immediately upon arrival to the laboratory, then needles were stored at -20°C. Volumetric moisture content (%) of the top 15 cm of soil was measured at the time of sample collection with a TDR instrument (Time Domain Reflectometry) using a 6050X3K1B Mini TRASE (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). The day prior to sample collection, soil apparent electrial conductivity of the top 160 cm

of the soil (ECa, mS s^{-1}) was measured within 20 cm of the base of each of the individual trees using a commercial magnetic dipole soil conductivity meter Dualem-1 (DUALEM, Milton, ON, Canada).

5.2.3 Sample processing

Roots were carefully isolated from sieved soil samples (250 μm) within 24 hrs of collection and washed with distilled water. Grass roots were removed from *P. radiata* roots, and obtained at least 1 g of *P. radiata* fine roots for each tree sampled. These root samples were weighed ($\pm$0.01 g) and kept wet at 4°C overnight for ECM colonisation measurements. To quantify the degree of ECM colonization, roots were cut into 1-cm pieces, arranged lengthwise in a thin layer along a gridline and examined under a dissecting microscope (Brundrett *et al.*, 1996). Between 100 and 250 intersections were assessed for each root sample and the proportion of ECM colonised root lengths was calculated as the percentage of intersects with mycorrhizal tips over the total number of intersects. The entire root sample was then homogenised under liquid nitrogen for DNA extraction.

Soil samples were homogenised, and 10 g subsamples were stored at -20°C for DNA extraction. Root length density (RLD, cm cm^{-3}) was calculated as root length by the volume of the total soil core. Soil pH was determined within 48 hrs of sample collection by shaking a 20 g subsample in 14 ml of 1 M KCl for 120 minutes and measuring the pH of the resulting extract (Miller & Kissel, 2010). Soil total N and C contents were measured with an elemental analyser (Elementar Isoprime 100 analyser, Isoprime, UK).

Needle water content was calculated as the percentage difference between fresh and dry foliage weight. Subsamples of needles and soil (200 mg) were dried at 60°C for 48 h and pulverised with a ball mill to a fine powder for nutrient analysis. Needle and soil N and C content were analysed with an elemental analyzer (Elementar Isoprime 100 analyser, Isoprime, UK), and needle δ^{13}C values were obtained after combustion, separation by gas chromatography and analysis by continuous-flow mass spectrometry (Europa Scientific 20/20 isotope analyser, Europe Scientific, Crewe, UK) at the University of Waikato (Hamilton, New Zealand). Carbon isotope discrimination (Δ ^{13}C) was calculated following the equation of Farquhar *et al.* (1989) that scales the δ^{13}C composition deviations from the Pee Dee formation reference material (δ_{air} = -8‰)

5.2.4 Community DNA isolation, Amplicon PCR and sequencing

The diversity and composition of bacterial communities in rhizosphere soil and fungal communities, in both roots and the rhizosphere, were characterised. From roots, DNA was isolated in triplicate from 150 mg of finely ground root tissue using cetyltrimethyl ammonium bromide (CTAB) buffer (3% CTAB 150 mM Tris-HCl, 2.5 M NaCl, 2 mM EDTA, 0.2% β-mercaptoethanol, pH 8). Rhizosphere soil DNA was isolated from 10 g sieved soil using the MoBio PowerMax Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA) following the manufacturer's protocol, but eluting in 500 μl Tris-HCl buffer. The concentration and quality of DNA extractions were assessed using a Nanodrop spectrophotometer (Nanodrop Technologies, Oxfordshire, UK). Samples were diluted in Tris-HCl buffer (pH 8) to equal DNA concentrations. When necessary, DNA extractions were concentrated prior to dilution (preciptation in 0.5 M NaCl and 100% cold isopropanol, followed by 70% ethanol wash). Bacterial 16S rRNA gene libraries were generated by amplifying the V3 and V4 region (approx. 460 bp) using the primers S-D-Bact-0341-b-S-17 forward (5'- CCTACGGGNGGCWGCAG -3') and S-D-Bact-0785-a-A-2 reverse (5'- GACTACHVGGGTATCTAATCC -3') (Klindworth *et al.*, 2013). For fungi, the target region was the internal transcribed spacer (ITS) region of the eukaryotic 18S rRNA gene (including ITS1, 5.8S and ITS2) amplified with the forward primer ITS1F-KYO1 (SSU) (5'- CTHGGTCATTTAGAGGAASTAA -3') and the ITS4 reverse primer (LSU) (5'- TCCTCCGCTTATTGATATGC -3') (White *et al.*, 1990; Toju *et al.*, 2012) generating estimated amplicon lengths of 500-600 bp. Forward and reverse primers had Illumina adapters (Nextera) ligated at 5' (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3' forward, 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -3' reverse). Amplicons were obtained in 20 μl reactions containing 1X KAPA HiFi HotStart ReadyMix, 0.3 M of each primer, and 10-15 ng of DNA template. Amplification was carried out with the following thermocycling protocol: 95°C for 3 min followed by 30 cycles; 98°C for 20 sec, annealing for 15 sec, 72°C for 30 sec, and a final extension 72°C for 3 min. In order to achieve a more robust estimate of microbial diversity, the amplicons from three parallel PCR reactions were pooled using 50°C, 55°C and 60°C annealing temperatures (Schmidt *et al.*, 2013). Amplicons were purified using the Agencourt AMPure kit (Beckman Coulter Inc., Beverly, MA, USA), and concentrations were determined with a Qubit Fluorometer (Invitrogen).

300-bp paired-end runs were undertaken on an Illumina MiSeq instrument at New Zealand Genomics Limited (NZGL, New Zealand).

5.2.5 Bioinformatic Analysis

Sequence processing was performed using the UPARSE pipeline (Edgar, 2013) (USEARCH v9.0.2132). Read 1 sequences were used for all communities, and fungal ITS reads from root and soil samples were processed together. Quality filtering of the reads was performed at maxiumum expected error (maxee) 1 (i.e. sequences with a predicted error rate of 1 nucleotide per sequence were discarded), followed by dereplication, and clustering of reads into OTUs based on a threshold of 97% sequence identity. The taxonomy of representative sequences for each OTU was assigned using the UTAX algorithm with the RDP Classifier (2016, trainset15) and UNITE (version 7.0) for bacteria and fungi, respectively (confidence threshold = 0.5). Samples with low reads (<100 reads), OTUs not classified at domain-level and OTUs with < 25 reads across at least 5 samples (Lundberg *et al.*, 2012) were omitted, keeping ca. 90% of the original reads. After sequence processing 20 samples for rhizosphere bacteria, 18 samples for root fungi, and 19 samples for rhizosphere fungi remained. A total of 489, 179 and 266 OTUs were observed across all samples for rhizosphere bacteria, root-associated fungi and rhizosphere fungi, respectively. For community analyses the relative abundances of OTUs were calculated in each sample by dividing the raw counts of each sample OTU by the sum of sample reads. After the bioinformatic analysis, FUNGuild v1.0 (Nguyen *et al.*, 2016) was used to classify fungal OTUs by ECM fungal guild.

5.2.6 Statistical Analysis

Data analysis was conducted in RStudio version 0.99.903 (RStudio Team, 2015) using primarily the vegan (Oksanen *et al.*, 2016) and ggplot2 (Wickham, 2009) packages. Principal coordinates analysis (PCoA) ordination was used to visualise relationships in soil physicochemical properties across N treatments and genotypes. The ordination was based on Euclidean distance and was performed with the *prcomp* function (stats package) applied to scaled and centered (with zero mean and unit variance) soil measures. Using the same distance measure, a permutational multivariate analysis of variance (permanova; Ander-

son, 2001) was conducted to test whether soils differed between tree N treatments or genotypes.

To test whether individual soil physicochemical properties differed between *P. radiata* genotypes and N treatments, two-way ANOVAs were conducted after confirming data met assumptions for normality and homogeneity of variance. When the interaction between genotype and N treatment was significant, differences among N treatments were tested for each genotype separately using one-way ANOVAs. *Posthoc* Tukey pairwise contrasts generated with the lsmeans package (Lenth, 2016) were used to determine which of the treatment combinations differed. The same approach was used to test for N treatment and genotype differences in plant characteristics, and OTU richness and Shannon diversity (H') for the rhizosphere bacteria, root fungi and rhizosphere fungi communities.

To test for relationships between soil physicochemical measures, plant growth measures and diversity of the root and rhizosphere communities, the significance of Pearson correlations was determined. Coefficients of Pearson correlations and p-values were calculated using the *corr.test* function (phsyc package, Revelle, 2015).

Multivariate relationships among the microbiota of individual trees were visualised with unconstrained principal coordinates analysis (PCoA) ordination plots using Bray-Curtis (OTU relative abundance) and Jaccard (OTU presence/absence) distance matrices. To test for differences in microbiota composition among N treatments and tree genotypes permanova tests (Anderson, 2001) were conducted with the *adonis* function (vegan package) for the Bray-Curtis and Jaccard distance matrices. As permanova tests cannot always distinguish between differences in multivariate location and dispersion (Anderson & Walsh, 2013), multivariate dispersion was also calculated with the *betadisper* function (vegan) and assessed significance with permutation tests (Anderson *et al.*, 2006). When the interaction between genotype and N treatment was significant, differences among N treatments were tested for each genotype separately with one-way permanovas.

To determine which soil physicochemical and plant growth measures were most associated with variation in the composition of the root and rhizosphere communities, a distance-based redundancy analysis (dbRDA) approach was applied. Bray-Curtis distance between samples for rhizosphere bacteria, root fungi and rhizosphere fungi communities was initially modelled with all plant and soil measures. The best model for each community

was determined with stepwise selection and applying 999 permutations using the function *capscale* of the vegan package (Oksanen *et al.*, 2016). Marginal tests were performed for all variables included in the final model.

5.3 Results

5.3.1 Rhizosphere soil properties

A principal components analysis ordination plot was generated to visualise relationships among samples in terms of measured soil rhizosphere physicochemical characteristics (Figure 5.1). While soil samples varied in their physicochemical properties, samples from trees treated with L-arginine tended to cluster separately from control trees and trees treated with NH_4NO_3 on PCo1 (Figure 5.1). Samples from the two genotypes tended to cluster separately on PCo2, especially under the L-arginine treatment. These patterns were confirmed by a permanova, which showed that soils differed between N treatments ($F_{(2,20)} = 5.27$, $p = 0.002$) and tree genotypes ($F_{(1,20)} = 6.45$, $p = 0.003$). There was no signficant interaction between N treatment and tree genotype for distance between samples based on soil physicochemical measures ($F_{(2,20)} = 2.36$, $p = 0.072$).

I assessed whether the individual soil parameters differed between N treatments or tree genotypes. The C:N of rhizosphere soil differed significantly between N treatments (Table 5.1). Soil C:N was significantly higher in the rhizosphere of control plants than plants that received L-arginine, while soil C:N of trees treated with NH_4NO_3 was intermediate and did not differ significantly from the control or L-arginine-treated trees (Table 5.1). There was a significant interactive effect of tree genotype and N treatment on soil pH. When differences among N treatments were tested for the two genotypes separately, soil pH was lower in the rhizosphere of genotype 31 trees supplied with L-arginine than in the rhizosphere of control trees. Rhizosphere soil pH in the NH_4NO_3 treatment of genotype 31 trees was intermediate and did not differ significantly from the control or L-arginine, while no differences were observed among N treatments for genotype 48 (Table 5.1).

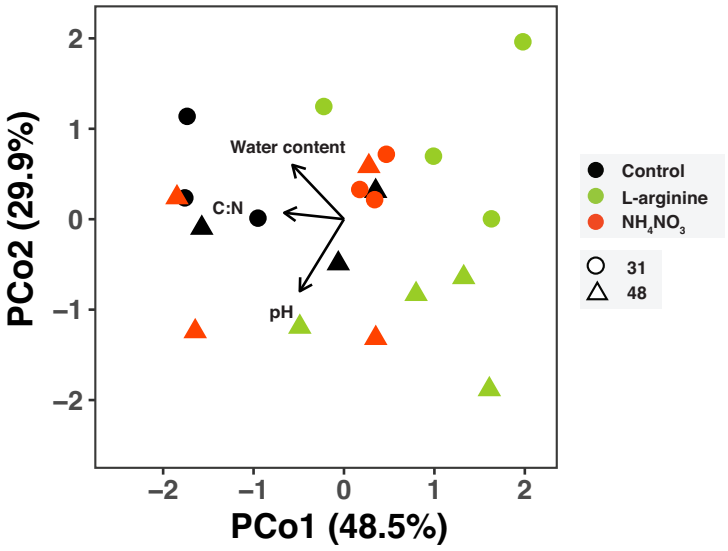

Figure 5.1: Principal coordinates analysis ordination biplot of distances between soil samples from two *P. radiata* genotypes (48 and 31) following two years of control, L-arginine and NH_4NO_3 treatments. The percentage of variation explained by each principal component is given in parentheses. Soil physicochemical properties are represented by vectors, and length and direction of vectors can be interpreted as correlations.

Table 5.1: Means (± standard deviation) of rhizosphere soil physicochemical measures. *F*- statistics and *p*-values from two-way ANOVAs testing for effects of N treatment, tree genotype and their interaction (G x N) on soil chemistry are given. Numbers in bold values indicate statistically significant results ($p < 0.05$). Letters indicate significant differences from Tukey *post hoc* tests comparing all pairs of treatment combinations and significant differences among N treatments when interactions were significant.

	Control		**L-arginine**		**NH_4NO_3**		**N treatment**		**Genotype**		**G x N**	
Soil properties	Gen. 31	Gen. 48	Gen. 31	Gen. 48	Gen. 31	Gen. 48	F	*p*	F	*p*	F	*p*
C:N content	11.42 ± 0.22^{a}	11.11 ± 0.52^{a}	10.58 ± 0.40^{b}	10.45 ± 0.25^{b}	10.61 ± 0.26^{ab}	10.86 ± 0.77^{ab}	4.50	**0.029**	0.07	0.789	0.63	0.543
C content	1.57±0.29	1.39±0.12	1.45±0.15	1.32±0.16	1.62±0.19	1.56±0.32	1.91	0.182	2.78	0.116	0.01	0.991
N content	0.14±0.03	0.13±0.01	0.14±0.01	0.13±0.01	0.15±0.02	0.14±0.02	1.57	0.239	1.61	0.223	0.14	0.871
Moisture content	5.86±0.57	4.80±0.81	4.63±0.84	3.71±0.82	5.05±0.43	5.24±1.33	3.68	0.050	2.35	0.146	1.02	0.384
pH	4.93 ± 0.06^{a}	4.91±0.09	4.61 ± 0.13^{b}	4.98±0.12	4.80 ± 0.01^{ab}	5.04±0.21	2.66	0.102	13.96	**0.002**	4.05	**0.039**
EC_a (mS s^{-1})	1.73±0.21	1.63±0.21	1.53±0.21	1.55±0.10	1.63±0.23	1.85±0.13	2.89	0.086	0.43	0.519	1.27	0.309

5.3.2 Tree growth and physiology measures

Next, I studied whether the N treatments had different effects on the growth and other physiological aspects of the two tree genotypes. Tree height differed significantly between genotypes, but not N treatments, with genotype 31 consistently taller than genotype 48 (Table 5.2). An interaction between N form and tree genotype significantly influenced needle C content (Table 5.2). To determine under which N treatments the genotypes differed in needle C content, differences between genotypes were tested for each of the three N treatments separately. Needle C content for genotype 31 was significantly higher than genotype 48 with NH_4NO_3 addition, while no significant differences between genotypes were observed for the control and L-arginine treatments. Needle C:N was significantly lower when L-arginine was supplied compared to control treatment. With NH_4NO_3 addition, needle C:N was intermediate and did not differ from either of the other N treatments (Table 5.2). Absolute needle water content also differed significantly between N treatments, with lower water content in needles from the NH_4NO_3 treatment than control or L-arginine treated trees (Table 5.2). In addition, needle water content was negatively correlated with soil N and C content (Table 5.3). Needle C isotope discrimination (Needle CID, a proxy for water use efficiency) and root length density were not influenced by either N form or tree genotype (Table 5.2).

The degree of ECM colonisation was significantly different between N treatments and tree genotypes (Table 5.2). Ectomycorrhizal colonisation was higher in roots of trees treated with L-arginine compared to control or NH_4NO_3-treated trees, and was consistently lower in genotype 31 than genotype 48. The degree of ECM colonisation was also negatively correlated with soil moisture content ($r_{(19)}$ = -0.61, p = 0.003; Figure C.2) and needle C:N content (Table 5.3).

Table 5.2: Means (± standard deviation) of plant characteristics. *F*- statistics and *p*-values from two-way ANOVAs testing for effects of N treatment, tree genotype and their interaction (G x N) on soil chemistry are given. Numbers in bold values indicate statistically significant results ($p < 0.05$). Letters indicate significant differences from Tukey *post hoc* tests comparing all pairs of treatment and genotype combinations and significant differences among N treatments when interactions were significant. [1]CID:Carbon isotope discrimination, [2]RLD: Root length density

	Control		**L-arginine**		**NH_4NO_3**		**N treatment**		**Genotype**		**G x N**	
Plant descriptors	Gen. 31	Gen. 48	Gen. 31	Gen. 48	Gen. 31	Gen. 48	F	*p*	F	*p*	F	*p*
Height (cm)	72.0±5.6^{a}	61.7±2.9^{b}	80.3±13.7^{a}	66.0±10.1^{b}	74.7±4.5^{a}	75.3±5.7^{b}	1.61	0.232	4.90	**0.042**	1.47	0.261
Diameter (mm)	19.1±2.3	14.6±0.9	19.2±4.0	17.8±2.8	17.0±1.4	19.3±1.1	0.87	0.439	0.97	0.339	3.03	0.078
Needle N content	1.22±0.05	1.32±0.08	1.36±0.06	1.38±0.10	1.30±0.09	1.36±0.04	3.21	0.069	3.13	0.096	0.43	0.655
Needle C content	48.87±0.19^{a}	48.86±0.23^{a}	48.71±0.20^{a}	49.00±0.18^{a}	49.00±0.29^{a}	48.67±0.09^{b}	0.13	0.877	0.01	0.974	4.65	**0.027**
Needle C:N	40.18±1.45^{a}	37.22±2.15^{a}	35.97±1.63^{b}	35.58±2.69^{b}	37.74±2.28^{ab}	35.75±0.99^{ab}	4.00	**0.040**	3.78	0.070	0.79	0.47
Needle CID[1] (Δ ^{13}C)	19.01±1.35	17.83±2.08	17.25±1.63	19.64±0.86	18.53±1.34	18.00±2.34	0.04	0.965	0.30	0.594	2.33	0.131
Needle water cont.	62.04±2.30^{a}	61.17±0.77^{a}	63.67±3.03^{a}	62.89±2.38^{a}	57.52±1.05^{b}	59.06±1.87^{b}	9.82	**0.002**	0.01	0.963	0.70	0.513
RLD[1] (cm cm^{-3})	2.19±0.63	3.24±1.45	3.14±1.05	2.95±1.05	3.67±0.84	1.81±0.60	0.41	0.671	0.79	0.385	3.62	0.051
ECM colonisation (%)	38.7±10.4^{c}	62.3±9.9^{abc}	74.0±11.6^{ab}	82.3±15.0^{a}	52.4±9.8^{bc}	56.6±7.0^{bc}	13.40	**<0.001**	5.57	**0.032**	1.37	0.284
Grass cover	16.7±7.6^{ab}	27.5±11.5^{ab}	12.5±9.7^{b}	15.0±11.0^{b}	25.8±10.1^{a}	39.4±4.3^{a}	8.60	**0.003**	4.43	0.052	0.72	0.501
Clover cover	26.7±12.6	27.5±11.5	27.5±20.1	38.8±9.8	25.8±10.1	34.4±13.3	0.33	0.721	1.51	0.238	0.26	0.776

Table 5.3: Relationships between above- and belowground components. Correlations between plant and soil descriptors and between plant variables and diversity were carried out using Pearson's correlations. Significant correlations are presented in bold (*$p < 0.05$, **$p < 0.01$). [1]Needle WC: Needle water content, [2]RLD: Root length density

	Height	Diameter	Needle N	Needle C	Needle C:N	CID	Needle WC[1]	Grass	Clover
Soil C:N	-0.129	-0.076	-0.28	-0.057	0.303	0.180	-0.141	0.140	0.003
Soil C	0.144	0.007	0.027	0.082	-0.006	0.245	**-0.453***	0.277	0.175
Soil N	0.171	-0.022	0.052	0.083	-0.034	0.226	**-0.491***	0.249	0.205
Soil moist.	0.115	0.063	-0.257	-0.072	0.262	0.051	-0.294	0.303	0.011
Soil pH	-0.257	-0.078	0.102	0.034	-0.076	0.196	-0.081	0.317	0.181
Soil Ec_a	0.151	0.265	-0.041	-0.176	0.026	-0.065	-0.318	0.188	-0.209
ECM colonisation	-0.158	-0.073	0.409	-0.109	**-0.426***	-0.097	0.244	-0.240	0.054
RLD[2]	0.081	-0.056	-0.111	0.161	0.101	-0.012	0.133	**-0.447***	-0.060
α-diversity									
Bacteria	-0.051	0.245	0.148	0.184	-0.132	0.131	0.386	-0.205	0.310
Rhizo fungi	-0.351	-0.288	0.325	0.422	-0.285	-0.077	**0.604****	-0.317	0.083
Root fungi	-0.303	-0.296	-0.077	0.218	0.108	0.326	0.129	-0.298	0.283
ECM fungi	-0.192	-0.020	-0.082	0.474	0.114	0.462	-0.131	-0.195	0.094

Tree genotype and N treatment significantly influenced the surrounding vegetation cover (Table 5.2). The percent cover of grasses was significantly different between N forms, with greater cover under the NH_4NO_3 treatment compared to the L-arginine treatment. In addition, the extent of grass cover was negatively correlated with P. radiata root length density (Table 5.3).

5.3.3 Rhizosphere bacterial community

Interactions between genotype and N treatment shaped the diversity and composition of bacterial communities in the tree rhizosphere. This interaction was significant for both observed OTU richness and Shannon diversity index (H') (Table C.1, Figure 5.2a-b). For genotype 48, OTU richness of the rhizosphere bacterial community was lower under control conditions than with L-arginine, while there were no differences in bacterial community richness among N treatments for genotype 31 (Figure 5.2a). Similarly, the Shannon diversity index was lower under the control treatment compared to the L-arginine treatment for genotype 48, with no differences detected for genotype 31 (Figure 5.2b). Principal coordinates analysis (PCoA) ordinations and permanova tests revealed that interactions between tree genotype and N treatment significantly contributed to variation in the composition of the rhizosphere bacterial community (Table C.2, Figure 5.2c-d). When differences among N treatments were assessed for each genotype separately, the bacterial rhizosphere community under L-arginine addition was significantly different from the control and inorganic N treatments for both genotypes (genotype 31: relative abundance of OTUs $p = 0.018$, presence/absence of OTUs $p = 0.039$; genotype 48: relative abundance of OTUs $p = 0.030$, presence/absence of OTUs $p < 0.001$).

Next, I investigated changes in the relative abundance of individual bacterial phyla when the mean relative abundance across all samples was greater than 1% (Figure 5.2e). The relative abundance of Bacteroidetes showed a significant interaction between tree genotype and N form (genotype x nitrogen: $F_{(2,14)} = 4.09$, $p = 0.039$), in which genotype 48 under L-arginine addition had a significantly higher relative abundance than with the NH_4NO_3 treatment (Tukey pairwise comparison; $p = 0.040$) (Figure 5.2e) and neither treatment differed from the control. In the rhizospheres of genotype 31 trees there were no differences in the relative abundance of Bacteroidetes. Tree genotype alone influenced

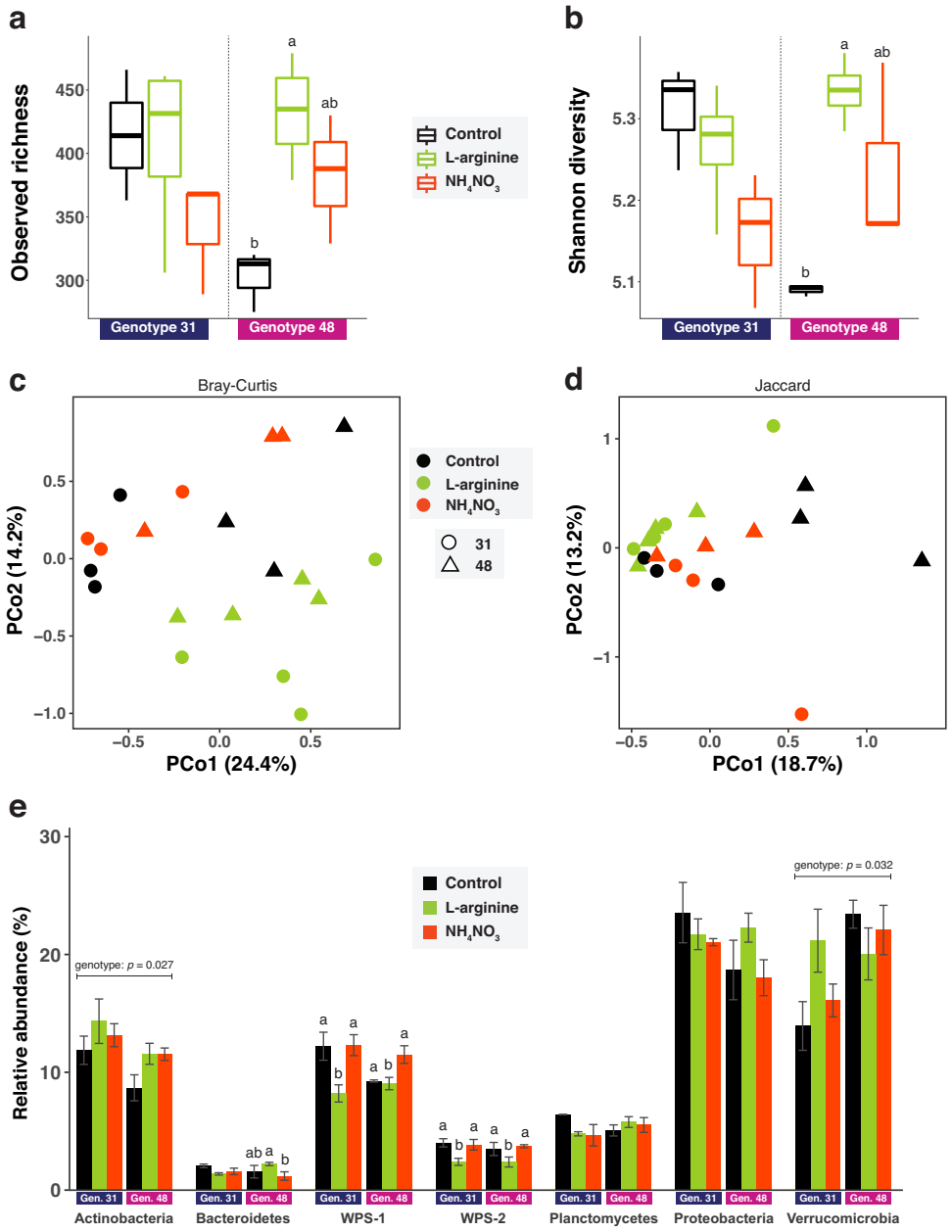

Figure 5.2: Shifts in bacterial communities in response to N treatment and host genotype (N = 20). (a) Diversity estimates for the effect of the interaction between genotype x N supply on community observed richness (α- diversity). The bottom and top edges of the boxes mark the first and third quartiles, the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above boxplots indicate significant differences across genotypes in response to N supply ($p < 0.05$) based on a *post hoc* Tukey pairwise comparison. (b) Shannon diversity index estimated for the effect of the interaction between N treatment and genotype. PCoA of Bray-Curtis (c) and Jaccard (d) distances between samples. (e) N treatment and genotype differences in the relative abundance of phyla-level taxa. Only taxa with mean of sample average greater than 1% in any of the N treatments are shown.

the abundance of Actinobacteria (genotype: $F_{(1,14)} = 6.01$, $p = 0.027$) and Verrumicrobia (genotype: $F_{(1,14)} = 5.69$, $p = 0.032$). The relative abundance of Actinobacteria was greater in genotype 31 than 48, while the relative abundance of Verrucomicrobia showed the opposite pattern between the tree genotypes (Figure 5.2e). N treatments differed in the relative abundance of candidate division WPS-1 (nitrogen: $F_{(1,14)} = 10.35$, $p = 0.002$) and candidate division WPS-2 (nitrogen: $F_{(2,14)} = 8.93$, $p = 0.003$). The relative abundances of candidate divisions WPS-1 and WPS-2 were consistently lower in the rhizospheres for both tree genotypes when treated with L-arginine compared to control (WPS-1, $p = 0.032$; WPS-2 - $p = 0.009$) or NH_4NO_3 (WPS-1, $p = 0.002$; WPS-2, $p = 0.007$) (Figure 5.2e).

5.3.4 Rhizosphere fungal community

Diversity of the rhizosphere fungal community did not differ significantly between tree genotypes or N treatments (Table C.1, Figure 5.3a-b). However, OTU richness of the rhizosphere fungal community was positively correlated with needle water content (Table 5.3). Principal coordinates analysis (PCoA) ordinations and permanova tests revealed that the composition of the rhizosphere fungal community differed between N treatments when distance between samples is calculated from OTU presence/absence, with no differences detected between genotypes (Table C.2, 5.3c-d). Differences among N treatments based on the relative abundance of OTUs was marginally significant. Differences in multivariate dispersion were likely to have contributed to these results as the composition of the rhizosphere fungal community with NH_4NO_3 addition was more variable than L-arginine ($F_{(5,13)} = 4.42$, $p = 0.014$, 5.3d).

The relative abundances of Ascomycota and Basidiomycota, the most dominant fungal phyla in the rhizosphere, were not significantly influenced by N treatments or tree genotype (data not shown). For fungal classes where the mean relative abundance across all samples was greater than 1%, several significant shifts were observed (Figure 5.3e). There was a significant interactive effect between tree genotype and N form on the relative abundance of Tremellomycetes (genotype x nitrogen: $F_{(2,13)} = 5.73$, $p = 0.016$). In the rhizosphere of genotype 48, the relative abundance of Tremellomycetes under the L-arginine treatment was greater than in the control ($p = 0.008$) or NH_4NO_3 ($p = 0.019$) treatments, while no significant differences were found for genotype 31. The relative

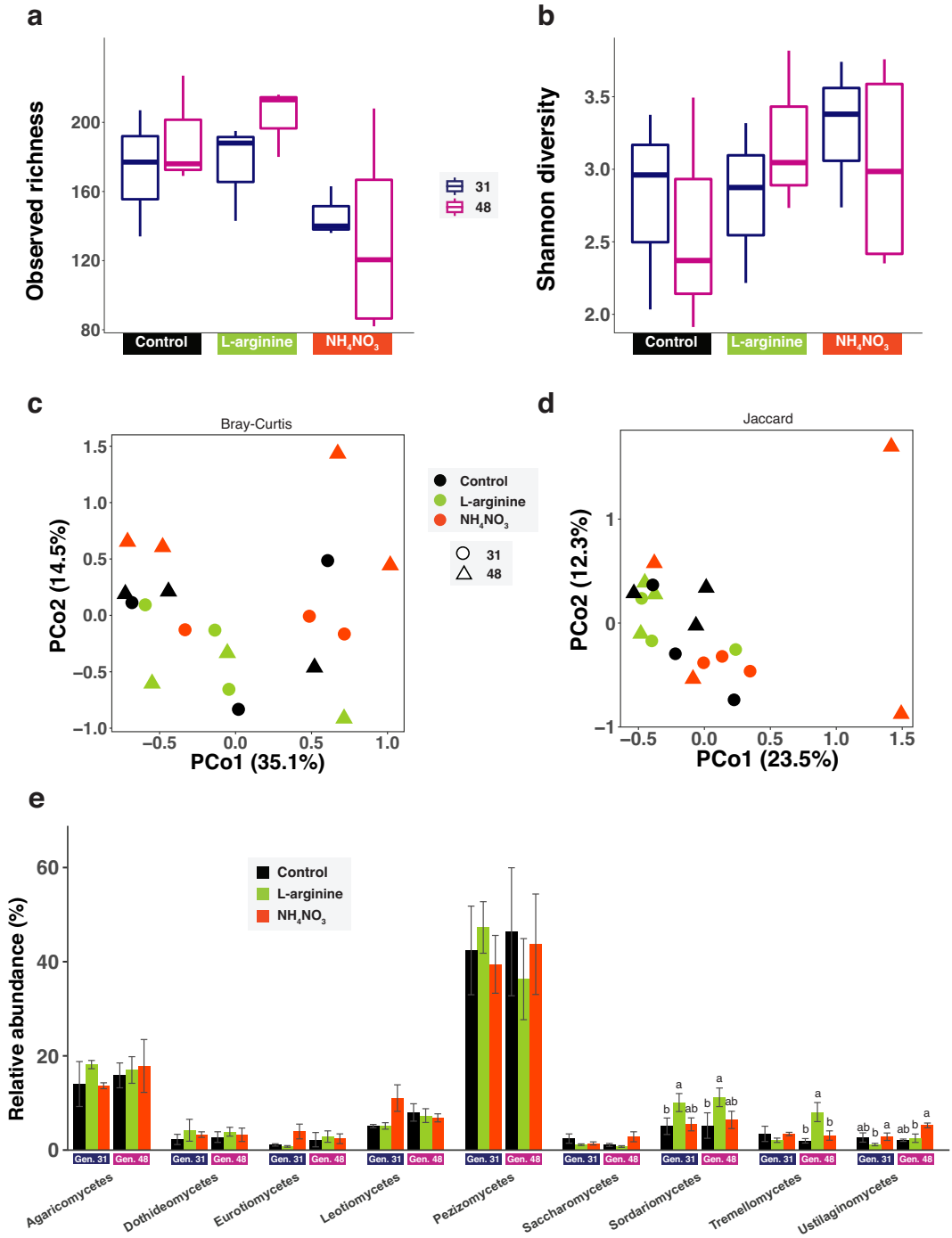

Figure 5.3: Shifts in fungal rhizosphere communities in response to N treatment and host genotype (N = 19). (a) Diversity estimates for the effect of N treatment supply among genotypes on community observed richness (α- diversity). The bottom and top edges of the boxes mark the first and third quartiles, the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above boxplots indicate significant differences across genotypes in response to N treatment ($p < 0.05$) based on a *post hoc* Tukey pairwise comparison. (b) Shannon diversity index estimated for the effect of N treatment and genotype. PCoA of Bray-Curtis (c) and Jaccard (d) distances between samples. (e) N treatment and genotype differences in the relative abundance of class-level taxa. Only taxa with mean of sample average greater than 1% in any of the N treatments are shown.

abundance of Sordariomycetes (nitrogen: $F_{(2,13)} = 4.36$, $p = 0.035$) and Ustilaginomycetes (nitrogen: $F_{(2,13)} = 7.31$, $p = 0.007$) responded to N treatment alone (Figure 5.3e). Greater abundance of Sordariomycetes was found with L-arginine addition than in the control ($p = 0.042$) or NH_4NO_3 ($p = 0.075$) treatments, and this response was consistent among genotypes. In contrast, the relative abundance of Ustilaginomycetes was greater with NH_4NO_3 addition than the L-arginine ($p = 0.014$) while no significant differences were found in the control.

5.3.5 Root-associated fungal community

The observed fungal OTU richness and the Shannon diversity index of the root-associated fungal communities did not differ between N treatments or tree genotypes (Table C.1, Figure 5.4a-b). Similarly, the composition of the root-associated fungal community was not influenced by N treatment or tree genotype (Table C.2, Figure 5.4c-d).

While there were no overall differences in the diversity or composition of the root fungal community, the relative abundance of some individual fungal taxa did differ between N treatments and host genotypes (Figure 5.4e). When N treatment and tree genotype effects were assessed for classes of root-associated fungi (with mean relative abundance across all samples greater than 1%), I found that the abundance of Tremellomycetes was significantly influenced by interactions between tree genotype and N form (genotype x nitrogen: $F_{(2,12)} = 3.93$, $p = 0.048$). The abundance of Tremellomyctes in genotype 48 was greater with L-arginine addition than with NH_4NO_3 addition ($p = 0.017$) but not different from the control treatment. No significant differences in the relative abundance of individual root-associated fungal taxa were observed for genotype 31. The relative abundance of predominant phyla Basidiomycota and Ascomycota in roots did not differ across tree genotypes or N treatments (data not shown).

I tested for N treatment and tree genotype effects on the community of root-associated fungi that were classified as ECM by FUNGuild (Nguyen *et al.*, 2016). There were no differences in alpha diversity measures for the root-associated ECM communities (Table C.1). Root-associated ECM communities differed significantly between genotypes when distance between samples is calculated from OTU relative abundance (Table C.2).

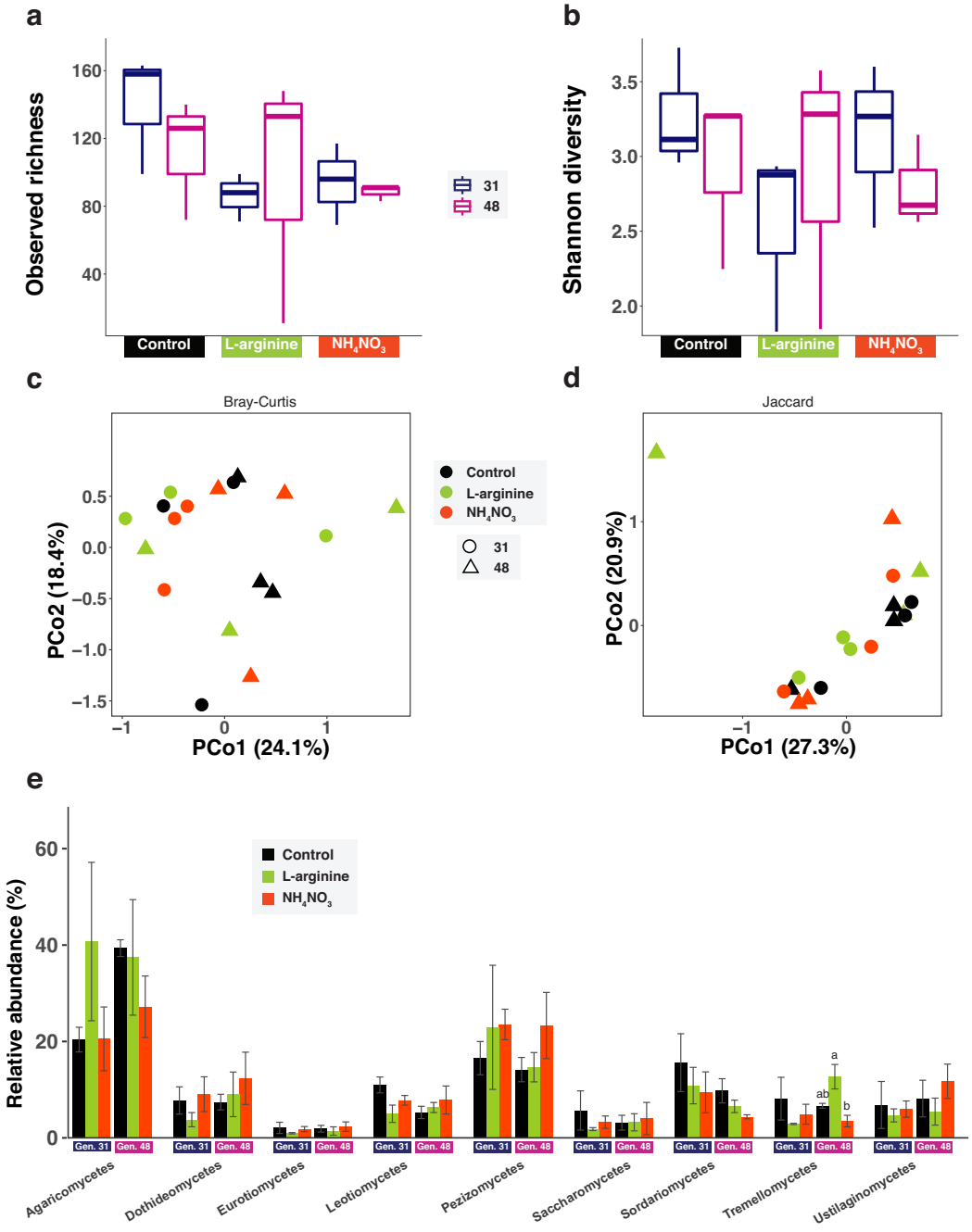

Figure 5.4: Shifts in fungal root-associated communities in response to N treatment and host genotype (N = 18). (a) Diversity estimates for the effect of N treatment supply among genotypes on community observed richness (α- diversity). The bottom and top edges of the boxes mark the first and third quartiles, the horizontal line within the box denotes the median and whiskers mark the range of the data. Letters above boxplots indicate significant differences across genotypes in response to N supply ($p < 0.05$) based on a *post hoc* Tukey pairwise comparison. (b) Shannon diversity index estimated for the effect of N treatment and genotype. PCoA of Bray-Curtis (c) and Jaccard (d) distances between samples. (e) N treatment and genotype differences in the relative abundance of class-level taxa. Only taxa with mean of sample average greater than 1% in any of the N treatments are shown.

The overall relative abundance of ECM fungi was not influenced by N treatment, tree genotype or their interaction.

5.3.6 Comparison of root and rhizosphere fungal communities

Of the 355 fungal OTUs detected across all rhizosphere and root samples, 225 fungal OTUs were unique to either rhizosphere or root-associated communities. The number of shared fungal OTUs between root and rhizosphere samples differed significantly between N treatments ($F_{(2,12)}$ =5.65, $p = 0.020$), but were not affected by tree genotype or the interaction between N treatment and genotype. The percentage of shared OTUs between root and rhizosphere samples was significantly lower in rhizospheres with NH_4NO_3 addition than in the control treatment ($p = 0.010$), while with L-arginine addition the shared OTUs did not significantly differ from the other treatments (Figure 5.5).

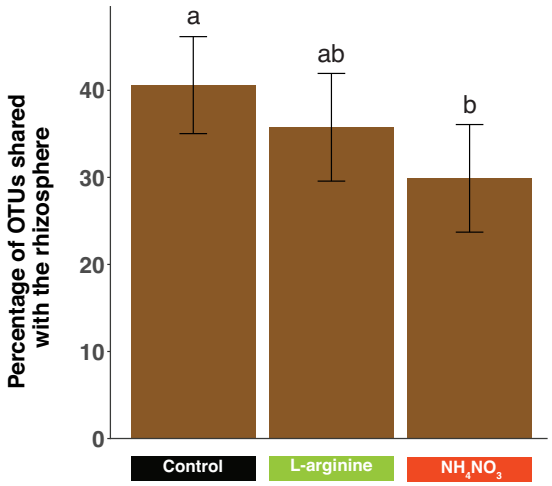

Figure 5.5: Effect of the N supply on the percentage of root fungal OTUs shared with the rhizosphere community. Letters above boxes indicate significant differences across genotypes in response to N supply.($p < 0.05$) based on a *post hoc* Tukey pairwise comparison.

The relative abundances of the shared OTUs in root and rhizosphere samples were positively correlated (analysis of covariance, $R^2 = 0.203$, $p < 2e^{-16}$). However, several significant differences were observed in the relative abundance of phyla and classes between

root and rhizosphere fungal communities (Figure 5.6). The phylum Ascomycota was dominant in the rhizosphere, and showed significantly greater relative abundance in the rhizosphere compared to roots ($F_{(1,35)} = 25.77$, $p < 0.001$). In contrast, Basidiomycota relative abundance in the rhizosphere was significantly lower compared to roots ($F_{(1,35)} = 27.37$, $p < 0.001$) (Figure 5.6). At class level, the relative abundances of dominant classes Agaricomycetes ($F_{(1,35)} = 13.97$, $p < 0.001$) and Pezizomycetes ($F_{(1,35)} = 29.99$, $p < 0.001$) significantly differed between rhizosphere and root fungal communities. The Agaricomycetes class was more abundant in roots, while the Pezizomycetes was more abundant in the rhizosphere (Figure 5.6). In addition, differences were found between the percentage abundance of less abundant classes Dothideomycetes ($F_{(1,35)} = 12.01$, $p = 0.001$), Saccharomycetes ($F_{(1,35)} = 4.17$, $p = 0.048$), Tremellomycetes ($F_{(1,35)} = 4.95$, $p = 0.032$) and Ustilaginomycetes ($F_{(1,35)} = 11.04$, $p = 0.002$).

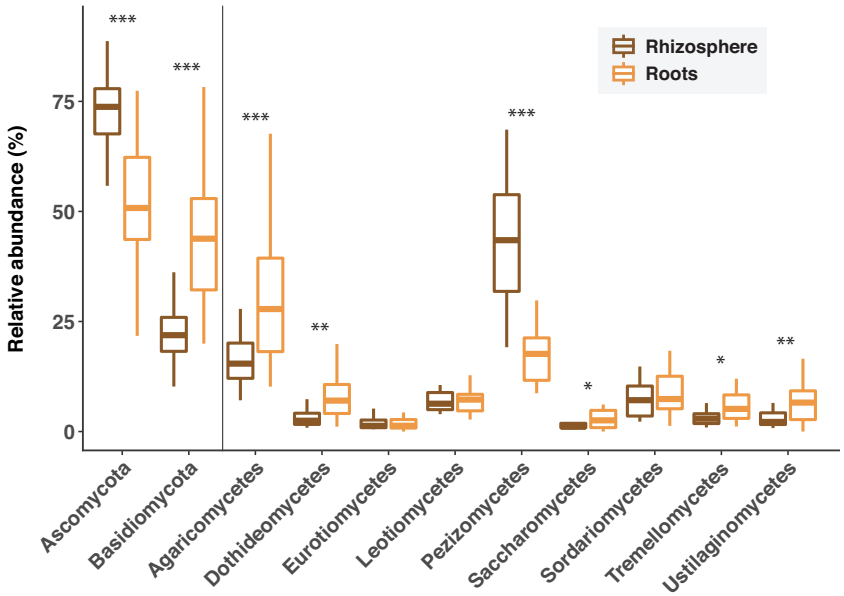

Figure 5.6: Relative abundance of phyla-level and class-level fungal taxa across rhizosphere and root-associated communities. Only taxa with mean of sample average greater than 1% in both communities are shown. Significance was assessed one-way ANOVA and asterisks represent the level of significance: * p < 0.05, **p < 0.010, *** p < 0.001.

5.3.7 Drivers of root and rhizosphere community composition

I applied a distance-based redundancy analysis model selection approach to determine which soil and plant parameters were most strongly associated with variation in the composition of the rhizosphere and root communities. For the rhizosphere bacterial community, soil C content, soil pH and needle N content were most significantly associated with variation in community composition. The most important factors for the rhizosphere fungal community composition were soil C content and needle N content. Marginal tests confirmed the significance for these explanatory variables individually (Table 5.4). In contrast, the measured soil physicochemical measures and tree characteristics were not associated with variation in the composition of root-associated fungal community (data not shown).

Table 5.4: Plant and soil variables predicting communities composition. *F*-models, *p*-values from marginal tests of distance-based RDA (db-RDA) modelling based on Bray-Curtis distance. Models were built considering all measured soil and plant variables (Table 5.1 and Table 5.2) and stepwise selection at a significance level $p < 0.05$. Adjusted R^2 correspond to the final model. Predictors used for the marginal tests were determined based on forward selection. Significant correlations are presented in bold.

	Soil C		**Soil pH**		**Needle N**			
	F model	*p*	F model	*p*	F model	*p*	R^2Adjusted	Full model
Bacteria	2.85	**<0.001**	1.91	**0.024**	1.77	**0.038**	0.149	**0.002**
Rhizosphere fungi	2.48	**0.021**	-	-	2.02	**0.045**	0.121	**0.010**

5.4 Discussion

While it is clear that belowground microbial and fungal communities influence aboveground productivity and other ecosystem functions, the underlying mechanisms that determine their composition remain unclear. Of particular interest are the impacts of genotypic variation in plant characteristics, soil conditions, and their interaction in response to different N sources. The findings show that both rhizosphere soil properties and tree physiology are influenced by tree genotype and the form of added N. Of the communities considered in this study (rhizosphere bacteria, rhizosphere fungi, and root fungi), rhizosphere bacterial communities demonstrated genotype-specific responses to N treatments for both diversity and composition. In contrast, differences among rhizosphere fungal communities were

driven primarily by N treatment, while root fungal communities showed little variation among tree genotypes or N treatments. Together, these results suggest that N-fertilisation with organic and inorganic N forms can affect rhizosphere communities within the root microbiome by (i) directly influencing plant responses, but also (ii) indirectly altering rhizosphere soil properies through genotype-driven responses. The investigation showed that N supplied in organic and inorganic forms can differently influence plant and rhizosphere soil nutrient cycling, changes that can involve shifts in the diversity and composition of rhizophere microbial communities. These findings are further evidence that interactions between plant genotypes and their root-associated communities are key determinants of soil nutrient availability and plant nutrient uptake capacity (Treseder & Vitousek, 2001; Koide *et al.*, 2014).

The addition of different N forms affected needle nutrient content, while variation among tree genotypes explained differences in growth. As shown by the PCoA ordination, soil properties for rhizospheres under NH_4NO_3 treatment were more similar to those under control than L-arginine. Interestingly, results also show that C:N of both rhizosphere soil and plant tissue decreased with the addition of L-arginine compared to the control, while adding an equivalent amount of N as NH_4NO_3 did not shift this ratio in either rhizosphere soil or tree needles. Recent studies have shown positive correlations between nutrient ratios in plant tissues and rhizosphere soil (e.g. Bell *et al.*, 2014), which indicate the presence of tight linkages between plant and soil nutrient cyclings. In consequence, changes in soil C and N dynamics can either influence or be influenced by rhizosphere microbial communities (Bever *et al.*, 2010) and favour microbial traits related to soil biogeochemical cycling processes via nutrient mineralisation/immobilisiation (Sistla & Schimel, 2012). The findings show that additions of different N forms can differently influence stoichiometry of plant needles and rhizosphere soil, and suggest this might be associated with the observed shifts in the microbial community composition. Of particular interest is whether differences in C:N ratios across N treatments were related to genotype-based changes in the N uptake capacity. Of all soil and plant variables, genotype influenced soil pH, plant growth and the ability to establish relationships with ECM fungi. Results are consistent with the evidence that N in organic form increases the mycorhizal colonisation (Turnbull *et al.*, 1995), possibly due to the high affinity of of mycorrhizal hyphae for amino acids

(Chalot & Brun, 1998). Variable ECM colonisation levels among genotypes were also observed. Genotype 31, which allocated greater resources to aboveground parts (Table 5.2), was generally less colonised, while genotype 48 allocated less biomass to shoots and had a higher colonisation rate. Although this study was non-destructive and it is not possible to confirm this pattern for the specific trees assessed in this study, differences in root morphological genotype-based traits could be driving differences in aboveground growth, through changes in the C invested for root growth, thereby influencing the proportion of C released by roots (Lamit *et al.*, 2016). Whether trees control soil nutrient ratios or the rhizosphere soil affected foliage nutrient ratios, I provide evidence that additions of amino acids, but not NH_4NO_3, might alter plant-microbe feedbacks and increase a genotype's capacity to take up N through changes in plant allocation of resources.

Rhizosphere microbial communities differed between N treatments and tree genotypes, but each of these communities (rhizosphere bacteria, rhizosphere fungi, and root fungi) had a unique response. The rhizosphere bacterial community was the only community influenced by interactions between tree genotype and N treatment, which suggests bacteria were impacted by variation in genotype-based traits, in contrast to rhizosphere and root fungal communities, which were less responsive to subtle changes among genotypes. In addition, a good predictor of the bacterial composition was soil pH, which was also influenced by the interaction between N treatment and genotype. The supply of organic N is often associated with an increase in soil proteolytic activity, through the exudation of proteolytic enzymes by either microbes, mycorrhizal fungi or plant roots (Bajwa & Read, 1985; Paungfoo-Lonhienne *et al.*, 2008). Proteolysis is strongly regulated by pH (Jones & Kielland, 2002), and studies have reported a negative correlation between soil pH and net arginine ammonification (Aciego Pietri & Brookes, 2008). Root-induced changes in soil pH can influence soil processes (Blossfeld *et al.*, 2011), such as mineralisation, which improve the availability of mineral N. Variation in soil pH in response to inorganic and organic N forms was more pronounced in the genotype with lower levels of mycorrhizal colonisation (genotype 31). This might be due to an increased root capacity to induce changes in rhizosphere soil pH in genotype 31 compared to genotype 48, possibly due to the lower reliance on ECM associations. In addition, soil pH was a strong predictor of the bacterial community composition. Differences in the abundance of Actinobacteria

and Verrumicrobia were mainly driven by tree genotype. Together with Proteobacteria, these taxa are generally in greater abundance in rhizosphere than bulk soil (Uroz *et al.*, 2010), and are often considered fast-growing *r-strategists* capable of utilising a wide range of root-derived C compounds (Philippot *et al.*, 2013).

The different responses of rhizosphere and root-associated fungal communities to N source possibly corresponded to position in a selective hierarchical process, ultimately driven by roots and root-induced changes in the rhizosphere (Bulgarelli *et al.*, 2013). The direct effect of N source on plant response was possibly the first filter determining the composition of the broader rhizosphere community, while those fungi inhabiting roots were likely more sensitive to genotype-specific local root responses. Further evidence of the filtering driven by N source were the several changes in the relative abundance of fungal rhizosphere classes (Sordariomycetes, Tremellomycetes and Ustilaginomycetes), while the composition of the root community showed very few shifts (Tremellomycetes). The significance of these selective forces on the fungal communities was further evidenced by the influence of inorganic N but not organic N (with the added presence of labile C) on the percentage of shared OTUs between root and rhizosphere samples, especially considering that diversity in the two communities was not altered by N treatment.

This continuum towards greater genotype selection as soil communities get closer to the influence of the plant, was also also true for the fungal guild more dynamically related to plant nutrition. The structure of the ECM fungal community was mainly influenced by host-genotype, and the levels of colonisation by ECM fungi were additionally influenced by N treatment. This suggests that despite N-fertilisation (high N availability), which is generally considered to negatively affect root colonisation by mycorrhizal fungi, here the supply of L-arginine induced greater ECM colonisation rates than in the control. Additionally, given the strong drought at the time samples were collected, the negative correlation between ECM and soil moisture content suggests that ECM had also an essential role in the uptake of water (Morte *et al.*, 2001). This agrees with the contrasting effect of N form on needle water content, in which trees under L-arginine had greater content of water in needles than under NH_4NO_3. Interestingly, root biotic interactions with ECM seem to influence needle water content much more than carbon isotope discrimination, which was not significantly different across genotype or N treatment, despite the fact that it is gen-

erally correlated with the efficiency of plant water use (Farquhar *et al.*, 1989). However, it is unknown if a greater association with ECM driven by N form has aboveground effects on needle gas-exchange parameters (Ruiz-Lozano & Azcon, 1995).

Soil C content and needle N content were significantly associated with variation in rhizosphere community composition. This highlights that C content in the rhizosphere had a determining role in the interactions between plant genotype and associated root microbiota in response to N form. As explained above, the additions of mineral N alone, but not when combined with labile C in the form of an amino acid, significantly decreased the shared fungal OTUs between rhizosphere and roots. Together, this suggests that a shift in the soil and plant C:N ratio driven by N additions can alter soil communities and associated processes. Although it is uncertain whether this was due to C release by microbiota or rhizodepositions, it demonstrates that C is a strong filter for recruitment, and C availability can be altered by the additions of different N forms. I hypothesise that the addition of the amino acid L-arginine stimulated a *rhizosphere priming-like* effect, naturally occuring in response to root exudation. Studies of the effects of exudate stoichiometry in a temperate forest through the supply of C + N compounds (Drake *et al.*, 2013), have shown an increase of SOM degradation and net N mineralisation, increasing the availability of N for plant acquisition. In contrast, exudates composed soley of C, enhance SOM degradation without promoting N mobilisation (Talbot *et al.*, 2008; Dijkstra *et al.*, 2013). The present study extend these findings and suggest that the addition of N + C in the form of amino acids, as opposed to N alone, might alter the rate and composition of root exudation and the association with ECM fungi, resulting in an increase of N availability. In contrast, the addition of N alone might lead to the consumption of N by microbes and plants with lower microbial recruitment required for the uptake of nutrients. I speculate that N-fertilisation with L-arginine (possibly as source of labile C) might serve as a form of communication in the interplay between roots and their associated biota.

5.5 Conclusions

The results provide evidence supporting the hypothesis that N form and tree genotype affect soil physicochemical properties as well as plant physiological responses and these responses can be extended to associated microbial communities in the root and rhizosphere.

The findings also show that rhizosphere communities (bacteria and fungi) were differently influenced by these factors. This suggests that tree genotype and N treatment differently regulate community assembly in a way in which, as communities are more closely associated with roots, they are more sensitive to genotype-induced changes. Moreover, N forms can differently influence stoichiometry of plant needles and rhizosphere soil, altering N and C dynamics in the rhizosphere. This likely promoted shifts in the microbial community composition and root-mediated changes in microbial recruiting. This study provides new progress towards a better understanding of the interacting genetic and environmental forces shaping root-microbe interactions and provides evidence that N-fertilisation with different N sources might differentially alter relationships between plant-genotype traits and root biota. Further research is required to understand the implications of plant-microbe feedbacks on soil nutrient availability and the underlying mechanisms driving the rhizosphere N and C flow in response to stoichiometric shifts or soil N composition.

Chapter 6

Summary, synthesis and implications

6.1 Key findings

6.1.1 Preamble

The main purpose of the present investigation was to contribute to the understanding of how genotype-by-environment interactions influence nitrogen nutrition of pines. This project aimed to investigate on-going questions regarding the significance of plant use of a broad range of plant-available N sources (amino acids), to progress beyond the vast majority of investigations that focus on plant uptake and assimilation of more reduced plant N-available sources (NH_4^+, NO_3^-), supported by the conventional view that plant N nutrition mostly rely on inorganic N sources. While an increasing number of studies have investigated species preference for different N forms, the significance of intraspecific variation in resource use, and how this influences phenotypic plasticity, is not well understood. This is particularly relevant in long-lived organisms, in which genetic-based traits may determine adaptability to changing soil C and N dynamics in forest ecosystems, and may help in predicting the outcomes of species competition in future environments. In the course of my thesis I studied how genetic variation develops in response to changing soil N environments (Chapter 2, Chapter 4) and how the resulting plant phenotypes affect the diversity and composition of the root-associated bacterial and fungal communities (Chapter 3, Chapter 5). The findings from these two broad questions show that amino acids, in this case L-arginine, can promote growth in young *P. radiata* trees as effectively as, and sometimes even more effectively than, inorganic fertiliser (NH_4NO_3) at the same N concentration. The results also show that the form of the N source differently influences above- and belowground plant traits among genotypes of the same species, that may directly and/or indirectly impact associated rhizosphere organisms. In light of the

major findings presented in this thesis, my research suggests that, while subtle changes in genetic background can to some extent predispose trees to *prefer* one N source over the other, organisms comprising the root microbiota (including bacterial and fungal taxa) are key determinants of soil N availability and plant capacity to access N sources. The key findings from the experimental studies carried out in each chapter are presented and discussed below.

6.1.2 Chapter 2

The main aims of the studies described in Chapter 2 were to explore if trait variation among genotypes of the same species plays a defining role in the acquisition and use of different chemical forms of available N. The investigation was carried out in greenhouse conditions and started by the screening of the aboveground growth responses among ten genotypes of *Pinus radiata* after 200 days of fertilisation with equimolar concentrations of different N sources: inorganic N (NH_4NO_3), organic N (L-arginine) and the combination of inorganic and organic N forms (L-arginine:NO_3^-). Three genotypes that exhibited the most contrasting responses in aboveground growth were selected, and three possible explanations for the variation were investigated based on root traits. This decision was taken based on recent evidence of variation in root morphology and root:shoot ratios in plants growing under organic and inorganic N (Cambui *et al.*, 2011; Lonhienne *et al.*, 2015). The studies tested (1) the genotype capacity to uptake inorganic and organic N form and its internal distribution, (2) the extent of root colonisation by symbiotic fungi, and (3) the area and morphology of the root-cross sections across root orders.

In a first experiment we examined if the selected genotypes of *P. radiata* had a preference for N form, and if this capacity varied according to observed changes in aboveground growth. The results I present indicate the presence of intraspecific variation in growth response to N form. While two out of ten genotypes showed significantly greater biomass under L-arginine than NH_4NO_3 supply, with varying responses to L-arginine:NO_3^-, among genotypes both N forms were taken up at broadly similar rates. A second experiment was designed to elucidate whether root biotic traits were related to the growth response, after quantifying the degree or ECM colonisation across root orders (proxy for root form and function, McCormack *et al.*, 2015). The level of root colonisation by ECM fungi was

significantly influenced by N source and negatively correlated with root order. However, among genotypes that were *responsive* and *non-responsive* to N form, no impact of host genotype was found. Roots from trees grown in organic N, solely or in combination with inorganic N, showed a consistently greater ECM root tip colonisation among genotypes. In the third experiment, we studied whether the root area and stele:cortex of the root-cross sections differed across genotypes. Regardless of the within-genotype variation, root area was greater in trees grown in L-arginine than those fertilised with NH_4NO_3, with a generally greater stele relative to cortex area. These results suggest that individual genotypes can benefit from the utilisation of one N source over the other, and achieve greater biomass, while roots exhibit relatively consistent measurable responses to N form regardless of genotype growth. These results provide evidence that plant growth is synergistically impacted by the interactions between the aboveground strategies of N use at the genotype level (i.e. metabolic strategies adopted) and belowground responses to the N environment (i.e. root plasticity).

Although the above results provide evidence of significant effects of N form between genotypes of the same species, the mechanisms underlying the differences in growth cannot be understood from the three experiments performed. These findings are broadly in line with previous research in conifers that indicate that the uptake rates for arginine and NH_4^+-N are of the same order (Gruffman *et al.*, 2013), although in the study NH_4NO_3 were both labelled, preventing an assessment of the relative contribution of the individual uptake rates. My findings are (to some extent) at odds with studies in conifer seedlings that indicate higher root-to-shoot ratios in plants supplied with arginine than with inorganic N (Cambui *et al.*, 2011), with no differences in total biomass. However, it is important to consider that the effects of altered biomass allocation in response to N form could be influenced by different rates of association with mycorrhizal partners. For instance, an increase in root growth might be compensated for by a greater uptake of that N form by mycorrhizae, which would explain the greater investment in shoot tissues. In line with this, my results are congruent with evidence of an increase in root absorptive area in response to organic N supply (Lonhienne *et al.*, 2015). This might be due to a greater contribution of ectomycorrhizal hyphae (Rousseau *et al.*, 1994), and the intrinsic increase in root area of individual roots. Of particular interest is the consistently greater

stele:cortex of the cross-sectional area in response to L-arginine compared to that under NH_4NO_3. A possible explanation of this, might be that the effect of L-arginine in tree roots increases root capacity for resources transportation, given the greater reliance on hyphal nitrogen absorption by the fungal partner. The ecological significance of such a response cannot be defined from this study, and so further experimentation is required at the whole-root system scale.

6.1.3 Chapter 3

In light of the findings from Chapter 2, in Chapter 3 we assessed the influence of tree genetics, N form and genetic-by-environment interactions on the composition of the root microbiome. Using plant and potting medium materials from the study in Chapter 2, two genotypes of *P. radiata* with contrasting physiological responses to exogenous organic or inorganic N supply were selected to undertake the study (genotypes 31 and 48). Genomic DNA from roots and potting media was extracted and processed to generate amplicon bacterial and fungal libraries by amplifying the bacterial 16S rRNA and eukaryotic 18S rRNA genes, respectively. After sequence processing and data analysis the rhizosphere communities (bacteria and fungi) and root-associated fungi (ectomycorrhizae and saprotrophs) of the two genotypes were characterised and compared.

Three main findings were obtained from the study. Firstly, diversity and composition of rhizosphere bacterial and root-associated fungal communities differed between genotypes that had distinct responses to N form. Secondly, shifts in the relative abundance of individual taxa were driven by the main effects of N form or host genotype. Thirdly, the root microbiome of the *P. radiata* genotype with the most divergent growth responses to organic and inorganic N was most sensitive to differences in N form. The results showed that intraspecific variation in tree response to N form has significant consequences for the root microbiome of *P. radiata*, demonstrating the importance of gene-by-environment interactions in shaping host-associated communities.

The findings support the hypothesis that feedbacks from host genotype presumably evolve as important factors determining the composition of the root microbiome. These results are in harmony with recent studies providing evidence that plant genotype is an important determinant of community assembly in the rhizoshere (Lundberg *et al.*, 2012).

Many other biotic and abiotic factors, such as soil physicochemical properties, have been reported to influence of the assembly of bacterial and mycorrhizal fungal communities in the rhizosphere (i.e. Fierer & Jackson, 2006). However, to-date, the relative contribution of these factors is unclear, and the intense variability across studies suggests that the assembly of the microbiota results from a hierarchy of events (Bulgarelli *et al.*, 2013), with edaphic factors being the stronger filter. However, considering that the investigation in Chapter 3 used material growing in highly controlled conditions with limited edaphic variability, the genotypic effects were possibly accentuated.

The more important findings in the study were the observed genotype-specific shifts in the root microbiome, and the possibility that this may be explained by distinct plant responses to N form. The results are broadly in line with the increasing evidence that variation in traits within plant species can drive differences in soil chemistry, with consequences on belowground microbial communities (Whitham *et al.*, 2006; Sthultz *et al.*, 2009). It is important to note that root-trait variation in response to N form (Chapter 2), in addition to what has been observed in previous studies (Lonhienne *et al.*, 2015), suggest that root traits might be strong candidates for the observed variation across the communities. Although this was not specifically tested in the study, this agrees with the increasing awareness that patterns of rhizodeposition and/or the rate of C transferred to the ectomycorrhizal partner may induce shifts in the composition or diversity of root-associated communities (Karst *et al.*, 2008; Baldi *et al.*, 2010).

The most significant result presented in Chapter 3, was that the tree genotype in which the growth response differed between N forms (genotype 31), also had the most divergent root microbiome responses to N form. According to recent studies, aboveground traits at the genotype level can significantly impact soil microbial communities and soil processes (reviewed by Schweitzer *et al.*, 2008a). Accordingly, it cannot be dismissed that differences in total biomass among genotypes, possibly driven by the variation in strategies of N use, would differently impact root communities. This broadly agrees with recent studies that propose linkages between the composition of the root microbiome with changes in tree productivity (Bazghaleh *et al.*, 2015) and plant fitness (Lau & Lennon, 2012). However, considering the present study is first of its kind, such connection cannot be drawn from the conclusions here.

6.1.4 Chapter 4

The study in Chapter 4 was designed to be complementary to that in Chapter 2. The experimental studies undertaken in both chapters used a similar experimental design, although the study in Chapter 4 was carried out in completely different conditions (field) and for a longer term (3 years). The main goal of Chapter 4 was to obtain aboveground growth responses in a plantation setting of the same ten commercial *P. radiata* genotypes to N-fertilisation using the same three different N sources in a silt loam soil. Given the fact that productivity of forest ecosystems is constrained by site resource availability, in addition to the utilisation at an individual tree level, we also assessed the impact of on-site environmental factors on this response. We compared, on an equimolar basis, the effect of N-fertilisation with inorganic N (NH_4NO_3), organic N (L-arginine) and the combination of both N sources (L-arginine:NO_3^-) to that of unfertilised trees. We measured tree height, diameter and site-specific descriptors of microsite variability (soil electrical conductivity and understory vegetation cover), and climatic information (rainfall and temperature).

After 2.5 years of fertilisation, we observed genotype-specific variation in the aboveground growth response to N source and this was significantly influenced by the field-scale heterogeneity in soil apparent electrical conductivity (EC_a) and the spatial distribution of the understory vegetation. The findings I present also provide evidence that inter-seasonal variation in precipitation and mean daily temperature influenced genotype growth increments in response to different N sources. These findings suggest that soil properties and associated environmental variables, in addition to climate fluctuations, are essential to the understanding of genotype performance and are crucial determinants of the intraspecific variation in response to N-fertilisation.

These findings corroborated what was observed in Chapter 2, and extended the significance of trait variation among genotypes of the same species in the utilisation of different forms of N to realistic field conditions. However, it is interesting that those genotypes with significant variation in response to 200 days of fertilisation to organic and inorganic N forms in the glasshouse (Chapter 2) differed from those that varied in response to 2.5 years of fertilisation with the same N source in the field (Chapter 4) (Figure 6.1). Many edaphic factors could drive these differences, such as the variable distribution in water and nitrogen resources driven by the spatial heterogeneity in soil properties and

understory vegetation - these are greatly minimised in the greenhouse experiment. In addition to the putative variation in N use postulated in the light of the findings in Chapter 2, fertilisation with different N source might also lead to changes in N availability driven by the heterogeneity in soil properties (EC_a) and the competitive impacts of understory weeds. In this, field-scale variation in water retention (EC_a) and microtopography might lead to changes in water availability that couple with variation in plant N availability. This suggests that N forms of distinct molecular structures experience different soil retention. Amino acids tend to move more slowly in the soil solution, due to interactions with clay surfaces (Kumari *et al.*, 1987) and generally show lower diffusion rates than NO_3 (Öhlund & Näsholm, 2002; Miller & Cramer, 2004). Hence, positively charged amino acids, like L-arginine, tend to easily bind to soil particles (Owen & Jones, 2001), and this has been primarily associated with a lower plant capacity to take up such nutrients. However, considering the soil type in this experiment was highly permeable, and that N additions were exogenously supplied to the base of the tree, I suggest that it might be advantageous for trees to have access to such N forms rather than highly mobile NO_3^-.

The somewhat surprising result was the overall lack of response to N source on aboveground growth compared to untreated trees. Recent studies have reported that treatments with organic N forms may contribute more efficiently than inorganic forms to plant N budget, even at lower concentrations, given the lower carbon cost of assimilation (Franklin *et al.*, 2017). Unfortunately, the experiment was restricted to limited measures of growth, and the lack of destructive sampling limits the knowledge of how the effects of N form and on-site variability influenced root responses. This makes it difficult to make direct comparisons with Chapter 2. Although these proposed explanations are likely sources of the variation observed, the mechanisms by which on-site variability can differently affect N fertilisation cannot be concluded from this study. In light of these findings, I suggest that management of forest plantations must thus consider the efficiency of N-fertilisation according to site-specific conditions in terms of biomass production and in the context of forecasted climate change scenarios.

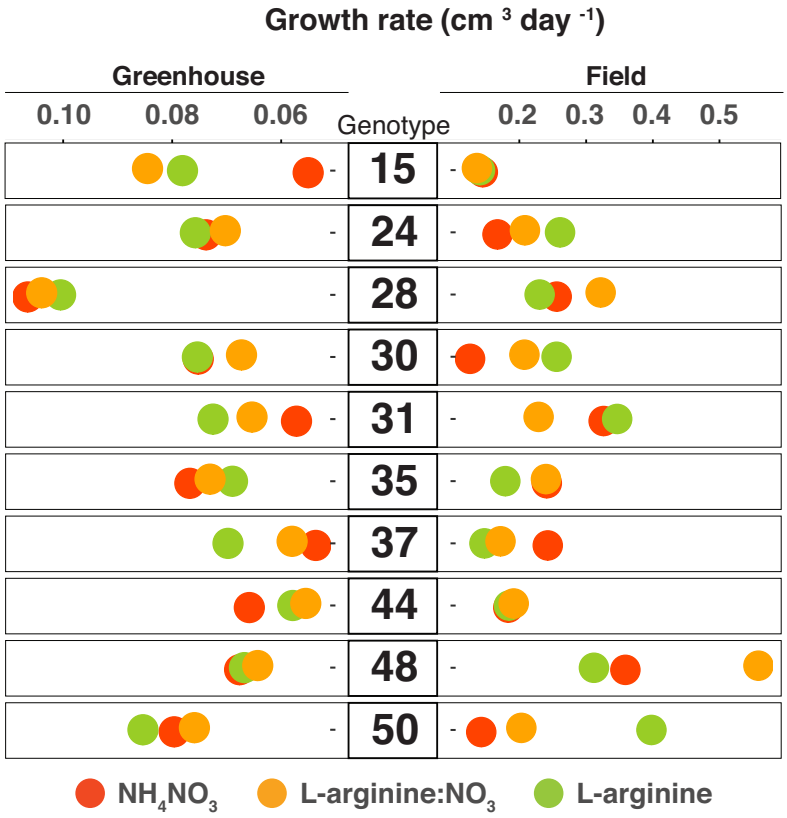

Figure 6.1: Comparison of volume growth rates in the greenhouse (6-month trial, highly controlled conditions) and field (2.5-years trial, no control of conditions) across the ten genotypes in this study.

6.1.5 Chapter 5

The main objective in Chapter 5 was to extend the understanding of genotype-by-environment interactions to host-associated communities under field conditions. The study characterised the rhizosphere bacterial and fungal communities, as well as root fungal communities of two full-sib *P. radiata* genotypes that previously showed distinct physiological responses to additions of organic versus inorganic N (Chapter 2 and 4). Similar to Chapter 3, the study aimed to understand how intraspecific variation in plant responses to abiotic conditions affects the diversity and composition of the rhizosphere, however in this case the investigation was carried out in the plantation setting studied in Chapter 4, two years after receiving yearly additions of NH_4NO_3 or L-arginine. Plant traits (height, needle nutrient content, water use efficiency, and ectomycorrhizal colonization rate) and soil physicochemical properties were measured for the same trees to link variation in tree re-

sponses to variation in rhizosphere and root communities. Using this approach, the study investigated whether variation in genotype-based traits in response to nitrogen form were influenced by soil physicochemical properties, and whether these effects were reflected in the rhizosphere.

The findings I present support the hypothesis previously postulated in Chapter 3, that distinct plant responses to N form influence host-associated belowground communities. In contrast to Chapter 3, the experiment was carried out in individuals with no differences in growth in response to N form, in order to minimise the effects of tree size on nutrient cycling. Three major findings emerged from the study. Firstly, both rhizosphere soil properties and tree physiology were influenced by tree genotype and the form of added N. Secondly, of the communities considered in this study (rhizosphere bacteria, rhizosphere fungi, and root fungi), rhizosphere bacterial communities demonstrated genotype-specific responses to N treatment for both diversity and composition measures. In contrast, differences among rhizosphere fungal communities were driven primarily by N treatment, while root fungal communities showed little variation among tree genotypes or N treatments. Thirdly, changes in the rhizosphere N and C dynamics, driven by genotype response to N form, had a determining role in the interactions between plant genotype and associated root microbiota. These findings suggest that N-fertilisation with organic and inorganic N forms can affect rhizosphere communities within the root microbiome by both (i) directly influencing plant responses but also (ii) indirectly altering rhizosphere soil properies through genotype-driven responses. The investigation showed that N supplied in organic and inorganic forms can differently influence plant and rhizosphere soil nutrient cyclings, changes that can involve shifts in the diversity and composition of rhizophere microbial communities. These findings are further evidence that interactions between plant genotypes and their root-associated communities are key determinants of soil nutrient availability and plant nutrient uptake capacity (Treseder & Vitousek, 2001; Koide *et al.*, 2014) Figure 6.2.

The main differences in the findings from Chapter 3 and Chapter 5 are the factors influencing rhizosphere and root-associated fungal communities, including the ECM fungal community. While the study under greenhouse conditions (Chapter 3) shows that G x N influenced root-associated fungal communities, I found no effect of any kind in the more

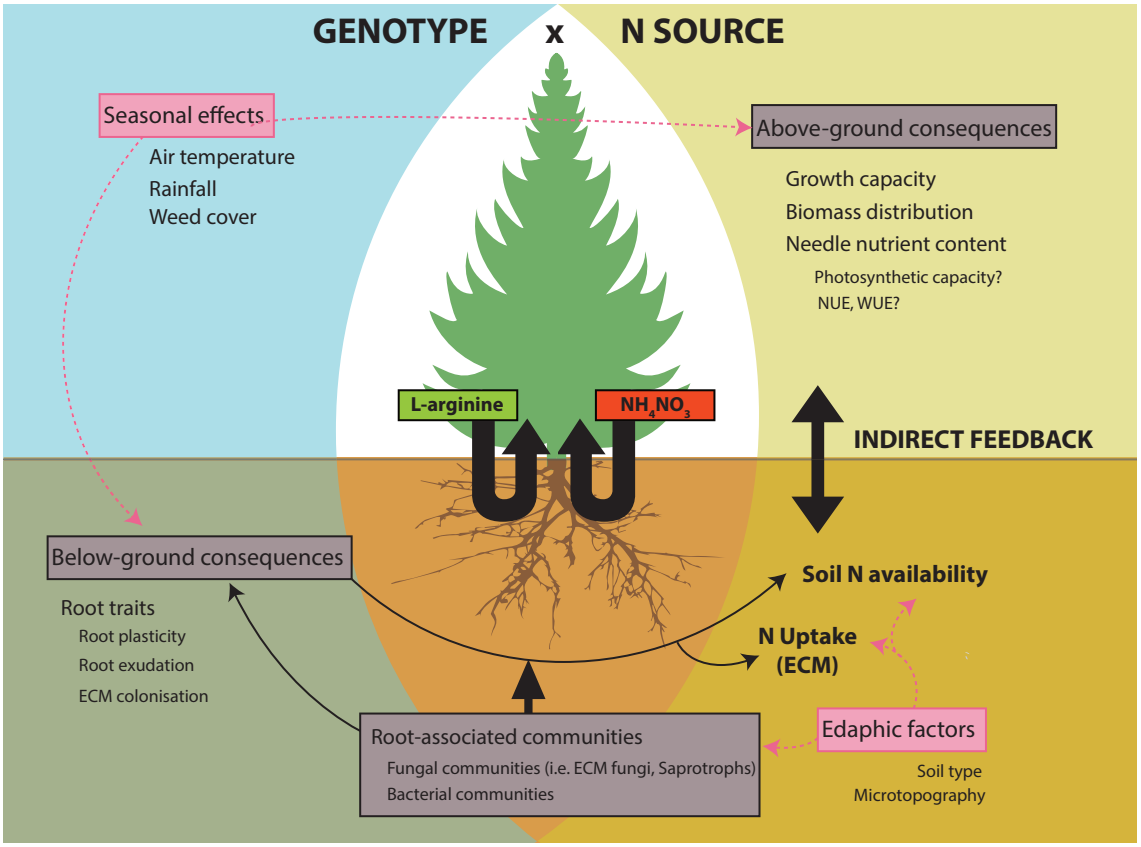

Figure 6.2: Conceptual model illustrating the interacting effects of both plant genotype and N source (L-arginine, NH_4NO_3) on plant responses, with a range of aboveground and belowground consequences that possibly affect soil N availability and the capacity to establish interactions with mycorrhizal fungi, which further facilitate the uptake of N. Tree genotype and N source impact the root-associated communities, including fungi and bacteria. Belowground communities can provide feedbacks to the host in response to aboveground and belowground plant responses. Consequences of fertilisation with different N forms can be further altered by seasonal effects and soil physicochemical properties (edaphic factors).

realistic field conditions. In addition, while in Chapter 3 the effect of G x N influenced the ECM communities, in the field (Chapter 5) such community was primarily driven by the effect of host genotype. As suggested in the discussion of Chapter 3 (section 6.1.3), these contrasting results obtained in very different growing conditions might be driven by differences in the effects of additional edaphic and climatic factors, and in fact these might be stronger filters (Bulgarelli *et al.*, 2013) (Figure 6.2). Interestingly, differences in the impact of N form on ECM communities, suggest that factors influencing ECM communities are somehow related with tree growth, as in each of the studies, the same factors that influenced the community influenced tree height. Similar to what was found in Chapter 3, organic N form increased root mycorrhizal colonisation (Turnbull *et al.*,

1995) and variable ECM colonisation levels were observed among genotypes. However, it is also interesting to note that one of the most obvious differences in growing conditions was the water supply - in highly controlled conditions water was provided by an automatic irrigation system, compared to the field conditions in which trees entirely relied on rainfall. This is particularly important for the potential impact of ECM community, which besides the facilitation of N uptake, may also have a role in plant water uptake in water-limiting conditions (Figure 6.2, Chapter 5).

The study in Chapter 5 allowed me to expand the study to question how different N forms impact soil physicochemical properties, in addition to plant physiological responses. In terms of the ecological significance, the study in Chapter 3 was constrained by the controlled conditions within a homogeneous potting medium with a consistent initial soil community. In contrast, the study of responses in a dynamic field system, overcame the shortcomings to understand whether plant responses were associated with rhizosphere soil nutrient cycling (Bell *et al.*, 2014). The results showed that soil properties for rhizospheres under NH_4NO_3 treatment were more similar to those under the control than under L-arginine. In addition, Carbon-to-Nitrogen ratios in both rhizosphere soil and plant tissue decreased with the addition of L-arginine compared to the control, while adding an equivalent amount of N as NH_4NO_3 did not shift this ratio in either rhizosphere soil or tree needles. The significance of the effect of plant and soil stoichiometry was related to the fact that soil C content and needle N content are strong predictors of variation in rhizosphere community composition, including bacterial and fungal communities. A possible consequence of this was that the additions of mineral N alone, but not when combined with labile C in the form of an amino acid, significantly decreased the shared OTUs between rhizosphere and root fungal communities, suggesting a lower microbial recruitment required for the uptake of inorganic N, but not for the uptake of organic N, compared to control conditions.

6.2 Implications, applications and future perspectives

Despite the limitations of fitting the study of a long-lived organism into a 3-year PhD programme, this thesis makes an important contribution across a number of areas of research. The following sections present the broader implications of the research in terms

of the expansion of knowledge and also suggest applications for some of the progress to improve forestry and agricultural ecology and management. In addition, and taking into account the key findings of the project, I introduce some suggested follow-up areas of research that would help understanding of tree N nutrition by incorporating the linkages between root trait-based uptake and use of different N sources, and the consideration that belowground communities are key determinants of the processes.

6.2.1 Understanding of G x E

The findings presented in this thesis emphasise that phenotypic variation exhibited within a subset of genotypes with narrow pedigree, can have far-reaching consequences, not only in terms of tree productivity, but also in terms of the capacity of shaping host-associated communities. *P. radiata* is a very well known species in New Zealand for its flexibility and adaptability to new environments, as seen by the increasing number of wild grown trees invading many different environments across the country (Richardson, 1998). In my opinion, this exemplifies the potential of *P. radiata* to exhibit a number of different phenotypes across small environmental variations and also supports the idea that a subset of genotypes can show highly distinct growth capacity. This variation is further significant considering that the set of genotypes came from progeny trials carried out in the same environmental conditions (Burdon, 1976) and based on similar selection criteria. This would explain the fact that genotypes with greater trait-variation in response to N source were full-sib. Of particular consideration, the phenotypic plasticity observed for *P. radiata* in response to N source might not be representative of other tree species with narrower ranges of developmental phenotypes. However, it is important to consider that the current study focused on N nutrition and variation in N-related traits might not be applicable to other plant traits, such as hardiness and water use efficiency. Nevertheless, within-genotype variation is a highly underrated factor in the study of model plant responses to environmental changes. This represents a potential source of variation that can lead to variance in findings when studying single species. The results from the current study urge the further study of variation across genotypes, ecotypes and cultivars of the same species, when these are available, in order to achieve more robust conclusions.

Gene-by-environment interactions have important implications for species evolution,

and this can have important implications for plant responses to environmental gradients and global climate change. In this study, genetic variance was generated by artificial selection from breeding progeny trials rather than evolutionary history. However, this research broadly contributes to the understanding of the range and determinants of responses amongst individuals within a species. The significance of such responses and the impact of the associated-organisms is especially important when working with long-lived plant genotypes. Trees exhibit greater plasticity than annual plants to cope with inter-seasonal changes in climate, and in the coming decades this capability is going be challenged, given the increasing progression of global climate change. Given the fact that forests ecosystems have a major role to play in the mitigation of human-driven climate changes, knowledge of tree responses to nutrient changes will be essential to predict tree fitness and forest ecosystem health.

6.2.2 Plant response to different N form

One of the main conclusion from this study is that tree N nutrition is a multi-factor and complex process that starts long before the uptake of nutrients. The observations in this study suggest that the exogenous supply of different N chemical forms can similarly promote tree growth. Furthermore, due to genetic and environmental factors, the supply of the amino acid L-arginine, solely or in equal combination with NO_3^-, can lead to significantly enhanced growth compared to NH_4NO_3. Genetic factors such as the metabolic strategies adopted by the genotype in a particular environment, can possibly be related to a different distribution of resources within the plant (as suggested by Cambui *et al.*, 2011). I propose that the observed plant response to N form was the product of the interaction between genetic factors of resource use and distribution with the phenotype exhibited in response to N form. Among genotypes, the consistent changes in root morphology and in the ability to establish symbiotic interactions with ECM fungi, suggest that the study of root morphological and architectural attributes should be the subject of future study. It is also quite important to elucidate how additional edaphic and climatic factors can further influence the tree response to N form, and whether these have a role in the immobilisation of nutrients in soil. Another physiological aspect that should be taken into account is the significance of leaf nutrient content changes in response to N form (Chapter 2 and

4). Further experimentation should determine if the variation in leaf characteristics to N source have an impact in terms of rates of gas exchange and photosynthetic capacity, and whether these are influenced by whole plant hydraulic conductance and associated tissue water potential.

From the findings in this thesis, the contribution of amino acids to tree growth strengthens the theory that depolymerisation of complex organic compounds to amino acids is a limiting step in plant available N-pool in ecosystems (Schimel & Bennett, 2004). It also suggests that phenotypic plasticity under changing soil N dynamics can have important implications at both plant and ecosystem scale (Whitham *et al.*, 2006). The effects of plant response to N form at a wider scale (beyond the plant) are far from being understood. Most of the studies undertaken to examine plant capacity to take up different N forms, alone or in combinations, are generally performed at a short-time and under unrealistic soil conditions (i.e. Gruffman *et al.*, 2014). After N additions, there is an initial peak of N concentration, followed by the progressive consumption of resources, either by plants or soil organisms, until resources are limited again. In natural ecosystems, this cycling is present in the form of N patches, although it might not be representative of how trees normally acquire nutrients. In addition, the presence of unique N forms in the rhizosphere environment is very unlikely, making it difficult to extrapolate my results to environments where the N forms present are not dominated by organic or inorganic N sources. Indeed, my work helps in the understanding, in a highly reduced model, of how trees acclimate - in the short-term - to large differences in the availability of N sources. Major developments is this field need both precise and affordable non-intrusive instruments to manipulate and measure changes in N forms in the soil solution, in order to expand our knowledge of temporal variation of soil nutrient cycling (Oyewole *et al.*, 2014). Identifying actual soil N composition would contribute to the understanding of plant preference and responses to N source (Inselsbacher & Näsholm, 2012).

Considering genotype variation as a mid-term evolutionary driver for adaptation, many questions remain unknown on how preferences for N form have evolved. Further research needs to untangle whether the use of a broader range of N sources has been a selection pressure throughout evolution, considering that some habitats are characterised by contrasting proportions of organic and inorganic N. If this is confirmed, it will provide

evidence that the use of inorganic N fertilisers in nurseries, especially in agricultural crops, has shifted natural selection by including breeding criteria which benefit those organisms with greater ability to take up and use inorganic N. In addition, knowledge should be expanded on the underlying processes involved in the advantages of using one form over another, and how this affects species and genotype development during the plant life cycle. In this regard, it is important to integrate the ecological perspective of rhizosphere communities to future investigations of plant nutrition, in order to contribute to a more complete understanding of the system. As shown by the early findings in Chapter 2, it is important to understand the mechanisms involved in the N form-driven root plasticity, and whether this serves as a functional strategy to increase the uptake capacity (transporters, higher absorptive are, ECM colonisation) or it is reflective of indirect effects of the relationships with the rhizopshere biota.

6.2.3 Forestry operations and genotype selection

The increasing demand for raw materials and the dwindling supply from natural forests has increased the importance of tree plantations (Payn *et al.*, 2015). In order to obtain highly productive forests by maximising yield and economic return, trees require silvicultural practices. N-fertilisation is a common practice in forest nurseries and after the establishment of plantations, and the N sources used are generally dominated by inorganic N fertilisers. Fertilisation has obvious advantages in terms of maximising aboveground growth, yet this happens at a cost. Ions such as NO_3^- have long been associated with leaching and low recovery in conifers, with important detrimental effects such as eutrophication and loss of biodiversity. Therefore, silvicultural practices must be adjusted to the tree species and the edaphic factors. In line with what was observed in Chapter 4, silvicultural operations require alternative N sources and adjusted regimes that can meet plant N demands, while minimising losses, according to the principles of sustainable development.

Sustainability in plantation productivity starts with the selection of those genotypes that can adapt to a wide range of conditions, while minimising the costs of management. The results in Chapter 4 emphasise the need for tree selection based on breeding criteria which expand the utilisation of a broader range of plant-available N pools across forest soils. Growth- and wood-related traits are multiloci, and are the product of coupling primary

and secondary metabolisms. Since the genetic resources in trees are still relatively scarce, it is necessary to increase understanding of selection criteria that are able to project a tree's adaptability to novel environments. This starts by carrying out phenotyping studies across environmental gradients. Having this information available would benefit the establishment of plant cultivars and genotypes, by improving the efficiency for plant nutrient acquisiton and utilisation. However, the availability of species or cultivars that are highly adaptive to heterogeneous resource distribution, with less dependency on reduced compounds, is still somewhat limited. In light of the recent evidence from low fertility areas in temperate regions, that free amino acids can contribute significantly to plant N available pools (Rothstein, 2009), it is important to further consider plant abilities to utilise organic N compounds. Finally, a step towards sustainable agricultural and forestry practices will not be achievable if the transference of scientific knowledge for breeders and land owners is not effective.

Productivity in the forestry industry equals stem growth. However, root traits (i.e. resource allocation to root growth) are critical to tree establishment and response to adverse conditions, which are both important drivers of tree mortality in a plantation settings. In line with this, the study of functional architecture of root systems would benefit genotype survival. We need to understand more about plasticity in root traits to environmental cues, especially in response to biotic and abiotic factors. Recent progress has contributed to understanding of the role of roots and root-induced changes to the rhizosphere. Roots are much more than a simple anchorage that scaffolds plant above-ground structures and absorbs water and nutrients - they are an active selector for those microbiome compositions, both prokaryotic and eukaryotic with both symbiotic and non-direct symbiotic roles, that are favourable for plant growth and survival. Unfortunately, in practice, nurseries are likely to have limited knowledge of this. The conventional operational procedures in nurseries tend to restrict the inoculation of young tree roots to sole or mixed spores of mycorrhizae species, most of which are generally unknown to the breeder. However, as suggested in Chapter 3, the diversity and composition of the ectomycorrhizal community can be strong determinants of tree productivity, association with particular mycorrhizal species can lead to more or less beneficial interactions.

6.2.4 Understanding of plant associated microbial communities

The results presented here provide evidence that intraspecific variation in plant responses to different N forms differently influences the diversity and composition of the rhizosphere community. Results suggest that N-fertilisation with organic and inorganic N forms can affect rhizosphere communities within the root microbiome by both (i) directly influencing plant aboveground responses (by changing the nature of plant-microbe interactions) but also (ii) indirectly altering rhizosphere soil properies through genotype-driven responses (by altering soil properties in ways that favour particular microbial taxa). In addition, Chapter 5 indicates that the response of genotypes to N source can differently select the bacterial and fungal organisms in the rhizosphere. The results have important implications in terms of elucidating the underlying mechanisms that determine the assembly of rhizosphere communities, and suggest that plant genotypes and their root-associated communities might differently influence rhizosphere N and C dynamics. In addition, temporal and spatial scales are of great importance in the rhizosphere of a developing tree. Therefore, it is also important to understand the eco-evolutionary drivers influencing the spatio-temporal shifts of plant-soil interactions.

It is really fascinating that roots, and their associated microbial communities, can act synergistically and not only impact plant development, but also impact nutrient availability. Although we don't know the exact extent under all systems, plant-microbe feedbacks have been shown to mediate soil biogeochemical cycles. This may have very important implications for plant N nutrition, in terms of how N forms transform and the factors influencing the abundance of plant N-available compounds. N availability is clearly driven by decomposition of soil matter. However, the factors influencing C and N transformation, the mineralisation/immobilisation of compounds and the factors influencing the rate and composition of rhizodeposition are still unclear. To make further progress, new technological approaches will need to emerge. The taxa present in the rhizosphere can now be efficiently characterized with next-generation sequencing approaches capable of recovering the majority of species in a sample with high-resolution. However, predictions of taxonomy are still poorly related to functional understanding. Although intra-domain interactions within soil communities have been fairly well studied, inter-domain have not been deeply investigated to date, despite their potential. Thereby, in cases in which factors differently

influence the diversity and composition of the rhizosphere communities (such in Chapter 3 and 5), not only the interactions between-communities but also within-communities would help to reveal additional indirect biotic factors able explain changes in bacterial and fungal community structures. Likewise, deeper functional knowledge that links ectomycorrhizal species, exploration types and morphotypes (Agerer, 2001; Koide *et al.*, 2014), would help understanding the range of resource trade-offs between plants and fungal partners in forest ecosystems.

In terms of sustainability, major effort is needed to elucidate the effects of N-fertilisation in order to identify the underlying processes diminishing soil and ecosystem health. Monoculture and agricultural practices tend to consider soil as inert, and that plant requirements should be exogenously supplied. Conversely, an increasing body of research has recently shown that the associated soil community can increase stress tolerance and improve plant productivity. Based upon this knowledge, future research and practice should increase crop reliability on soil beneficial taxa thay promote plant health and growth from germination onwards. Although thousands of plant growth-promoting bacterial strains have been isolated, the mechanisms by which they operate to increase yield is still somewhat of a black box. Plant breeders and crop and plantation managers needs to incorporate such benefits to establish sustainable management of crops and plantations that minimises inputs but ensures sustainable improvements in yield into the future.

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Appendix A

Supplement chapter 3

A.1 Supplementary Information

Taxonomic assignments (confidence = 0.8) at the bacterial domain accounted for 92.82% of the filtered OTUs (363,955 reads (98.15%)), 1748 at phyla level (343,567 (92.65%)), 1154 at class level (259,738 reads (70.04%)), 495 at order level (116,178 reads (31.33%)), 264 at family level (50,922 reads (13.73%)) and 72 at genus level (12,217 reads (3.29%)). No Achaea OTUs were found using RDP-Classifier taxonomy database. The most abundant bacterial phyla represented on the 2062 bacterial OTUs obtained was Proteobacteria (579 OTUs/28.1%) followed by Planctomycetes (183 OTUs/8.9%), Parcubacteria (155 OTUs/7.5%), Acidobacteria (149 OTUs/7.2%) and Bacteroidetes (143 OTUs/6.9%).

After the OTU clustering and the taxonomic prediction (confidence = 0.8) fungal communities accounted for 220 OTUs in root (577,451 reads (80.06%)) and 347 OTUs in soil samples (722,459 reads (75.35%)) at phyla level, 109 (360,577 reads (49.99%)) and 185 (524,963 reads (54.72%)) at class level, 81 (276,074 reads (38.28%)) and 132 (327,859 reads (34.18%)) at order level, 51 (263,544 reads (36.54%)) and 81 (271,328 reads (28.28%)) at family level and 20 (50,339 reads (6.98%)) and 30 (60,394 reads, 6.30%) at genus level, in root and soil samples respectively. 88 OTUs of the total OTUs obtained for the fungal community were discarded for not belonging to the fungi domain with a minimum confidence threshold of 0.8. The abundance of fungal reads across phyla in roots and soil samples respectively, were distributed in Ascomycota (163 OTU/60.5% reads and 254 OTU/42.9% reads), Basidiomycota (48 OTUs/17.3% and 75 OTUs/28.6%), Zygomycota (7 OTU/0.7%, 14 OTU/1.4%), Chytridiomycota (2 OTUs/1.0% and 4 OTUs/1.17%) and 1 Glomeromycota OTU (<0.1% soil reads) only present in soil samples.

The prediction of fungal guilds using the FUNGuild classification assigned the 35.5%

of the root fungal reads (256,254 reads) to be ectomycorrhizal (ECM) fungi, and OTUs were distributed in Ascomycota (11 (67659 reads)) and Basidiomycota (6 (64,421 reads)). The dominant class was Agaricomycetes (Basidiomycota) (4 roots (63,526 reads)), followed by Sordariomycetes (Ascomycota) (3 roots (6,606 reads)). The saprotrophic community represented the 5.8% sequences of the total present in root samples, and was represented by a higher number of OTUs comparing to ECM fungi. Sequences were also distributed between Ascomycota (75 roots (19231 reads)) and Basidiomycota (23 roots (12811 reads)). Agaricomycetes was also the dominant class (9 roots (10292 reads)) followed by Eurotiomycetes and Sordariomycetes, in order of abundance.

A.2 Supplementary Material

Table A.1: Plant characteristics measured in genotype 31 and 48 in response to NH_4NO_3 (inorganic N) and L-arginine (organic N) supply. Averages (mean with one standard error) were calculated considering 8-9 replicates from each condition. Two-way ANOVA results are provided as F value (p value) and significant p values are highlighted in bold.

	Clone 31		**Clone 48**		**Two-way ANOVA**		
	Inorganic N	Organic N	Inorganic N	Organic N	N form	Genotype	N x G
Tree height (cm)	24.9±3.4	30.1±1.8	26.3±2.4	25.3±2.5	3.72 (0.061)	0.01 (0.937)	1.99 (0.166)
Tree diameter (mm)	10.8±0.2	11.3±0.2	11.1±0.3	10.8±0.3	0.01 (0.967)	0.04 (0.836)	1.64 (0.208)
Total biomass (g DW)	58.5±2.7	71.8±4.3	70.1±5.0	64.6±3.8	0.53 (0.473)	0.44 (0.512)	4.82 (**0.035**)
Stem biomass (g DW)	13.6±0.9	18.1±0.9	16.2±1.2	15.9±1.2	2.85 (0.100)	0.12 (0.725)	4.39 (**0.043**)
Foliage biomass (g DW)	24.8±0.7	30.1±1.6	26.3±1.2	25.3±0.9	2.76 (0.105)	1.38 (0.247)	6.40 (**0.016**)
Total leaf area (cm^2)	2405.9±258.3	2362.5±485.9	1796.9±131.9	1784.6±391.7	2.60 (0.145)	4.90 (0.058)	0.07 (0.794)
Needle N content (g N g^{-1} DW 100)	1.06±0.04	1.14±0.04	1.11±0.04	1.20±0.04	4.25 (**0.045**)	1.55 (0.219)	0.01 (0.912)
Needle N:C ratio (g N g^{-1} C)	0.019±0.001	0.021±0.002	0.020±0.001	0.022±0.002	1.54 (0.248)	0.14 (0.719)	0.01 (0.994)
Root biomass (g DW)	19.9±1.7	23.6±2.2	27.5±3.4	23.3±1.9	0.10 (0.755)	2.15 (0.151)	2.23 (0.144)
N uptake rate (nmol N g^{-1} h^{-1})	0.11±0.02	0.09±0.01	0.15±0.04	0.12±0.02	0.65 (0.427)	1.50 (0.230)	0.01 (0.906)
ECM colonisation (%)	51.8±2.6	65.8±4.5	42.6±2.27	53.2±1.2	17.57 (**0.003**)	13.95 (**0.006**)	0.33 (0.580)

Table A.2: Properties of the potting mix used for the experiment. Descriptors of the properties were obtained at the end of the experiment and expressed as minimum-maximum (mean).

Properties	
pH (KCl)	4.10-4.70 (4.34)
Moisture content (%)	34.77-55.70 (48.05)
N content (%)	0.250-0.420 (0.292)
C content (%)	24.67-33.76 (30.25)
Composition	15% bark
	50% pine fines
	15% cocoa fibre
	20% pumice

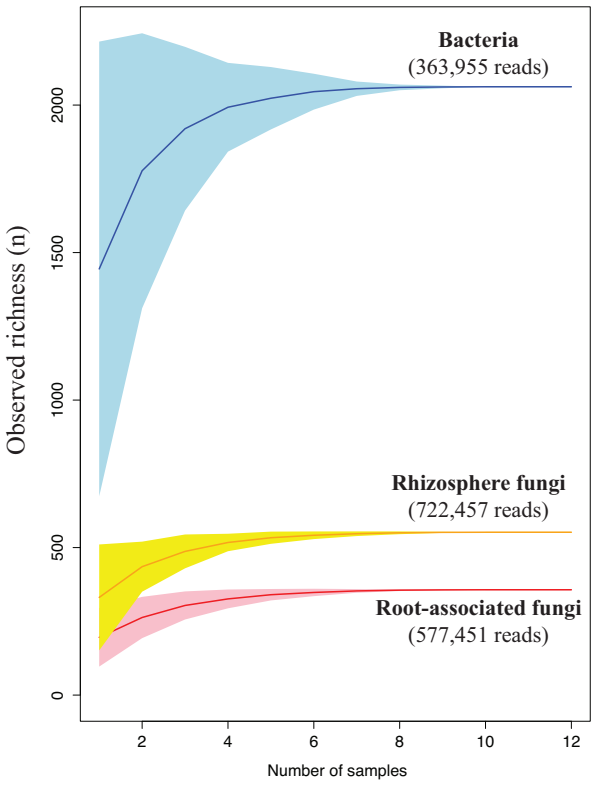

Figure A.1: Sample-based species accumulation curves for bacterial OTUs (2,062), rhizosphere fungal OTUs (552) and root-associated fungal OTUs (357) on tree microbiomes across the entire experiment. Shades on each curve represent the 95% confidence interval.

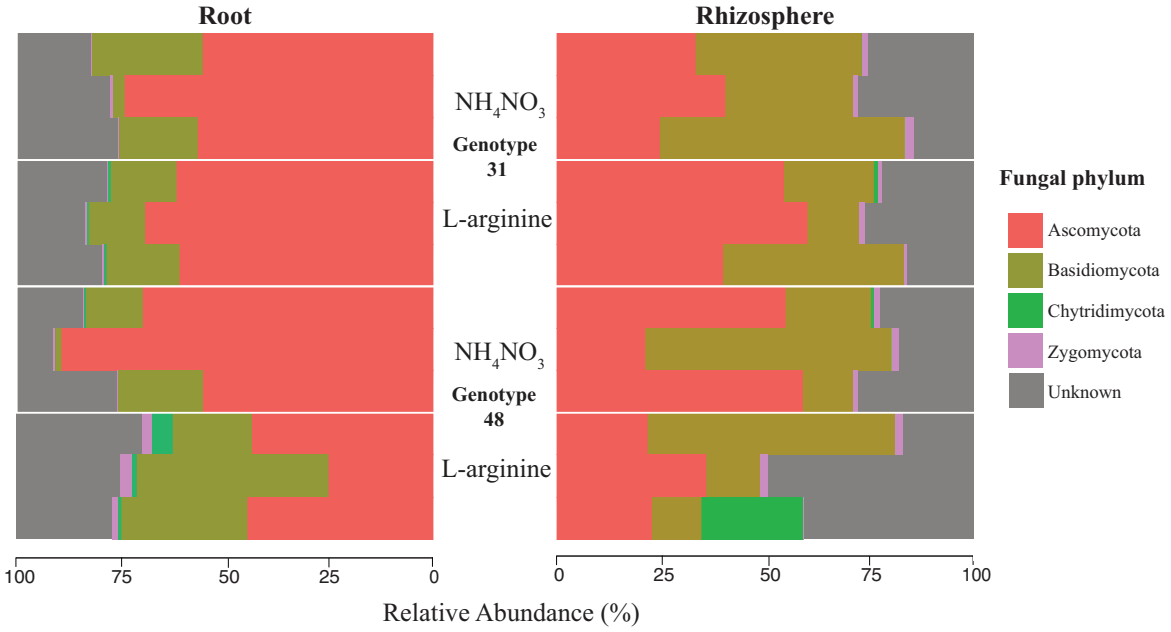

Figure A.2: Relative abundances of fungal phyla in root and rhizosphere samples from three independent replicates corresponding to genotypes 31 and 48 in response to NH_4NO_3 (inorganic N) and L-arginine (organic) N fertilisers.

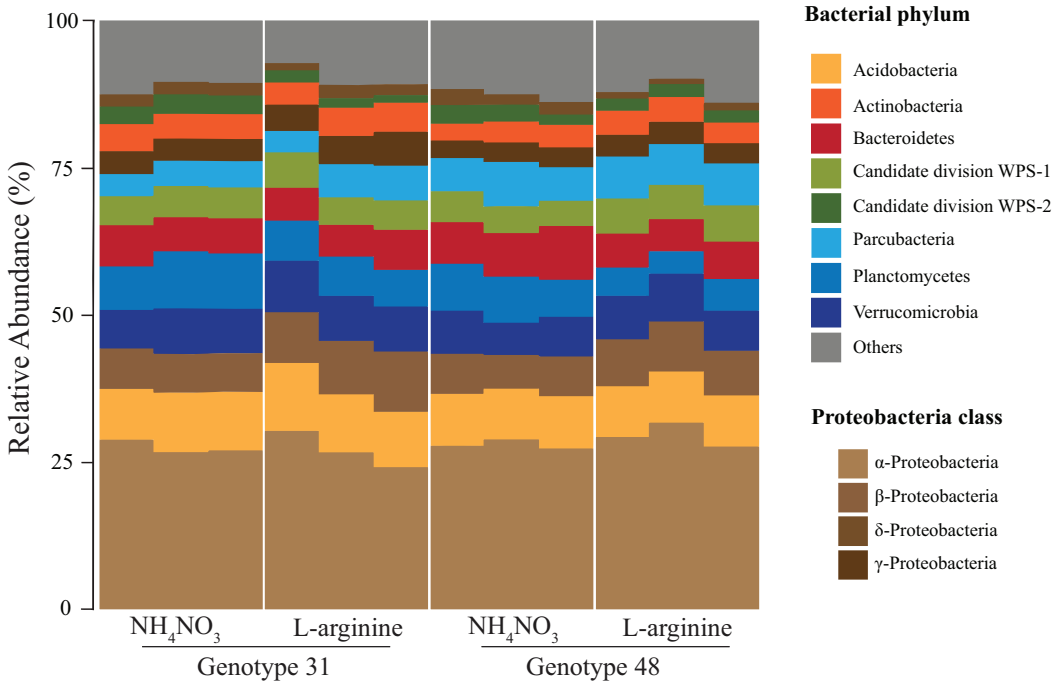

Figure A.3: Relative abundances of bacterial phyla, including Proteobacteria classes, for three independent replicates corresponding to tree genotypes 31 and 48 in response to NH_4NO_3 (inorganic N) and L-arginine (organic) N fertilisers.

Appendix B

Supplement chapter 4

Table B.1: Climate data of monthly cumulative precipitation (mm), daily minimum temperature (°C), daily maximum temperature (°C) and relative humidity (%) between 2013 and 2016. The top values represent the average historic record [mean ± SEM] between 1972 and 2015. Data obtained the Darfield NIWA weather station (agent 4836, 43°29'S 172°08'E).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Des
Precipitation												
1972-2015	55±41	55±32	65±50	64±48	67±40	72±48	72±45	81±52	56±37	69±35	58±30	67±39
2013	-	-	-	-	-	-	-	-	43.4	72.8	31.8	81.0
2014	15.0	57.0	150.4	216.6	47.2	98.0	0.0	17.0	29.4	20.0	44.2	26.2
2015	17.6	10.8	0.0	59.6	15.6	72.6	44.4	41.4	52.4	11.4	16.2	46.6
2016	87.2	18.6	47.6	12.8	84.0	21.0	-	-	-	-	-	-
Minimum temperature												
1972-2015	11±1	10±1	9±1	7±1	4±1	2±1	1±1	2±1	4±1	6±1	7±1	9±1
2013	-	-	-	-	-	-	-	-	3.6	5.7	9.0	11.1
2014	9.7	11.7	8.7	9.1	3.3	1.8	0.9	1.0	2.6	4.6	6.8	10.3
2015	12.5	10.1	10.5	7.7	2.9	0.5	0.3	1.7	3.2	5.0	7.5	9.2
2016	12.2	13.2	10.1	5.5	6.3	2.2	-	-	-	-	-	-
Maximum temperature												
1972-2015	23±2	23±2	21±2	18±1	14±1	11±1	10±1	12±1	15±1	17±1	20±1	22±2
2013	-	-	-	-	-	-	-	-	14.3	17.7	18.9	21.7
2014	22.2	21.9	18.6	15.6	15.7	13.1	11.2	11.9	14.3	17.3	20.0	21.4
2015	24.0	22.5	20.3	18.2	16.2	12.9	11.4	11.8	13.4	18.2	19.4	21.8
2016	20.8	25.1	21.3	18.9	17.2	13.7	-	-	-	-	-	-
Relative humidity												
1972-2015	64±6	69±5	70±6	74±5	75±6	76±6	74±5	73±5	71±6	70±5	69±5	68±6
2013	-	-	-	-	-	-	-	-	78.4	71.0	76.9	69.2
2014	63.8	72.2	81.0	87.6	74.4	78.5	78.0	77.4	83.2	72.6	60.1	73.4
2015	66.9	69.2	76.1	74.3	70.2	71.3	70.6	78.9	76.2	68.8	63.3	65.9
2016	73.0	67.4	72.9	79.0	69.6	75.0	-	-	-	-	-	-

H

Appendix C

Supplement chapter 5

Figure C.1: Summary of experimental design. (a) Map of the study region in Canterbury, New Zealand. Samples were collected from a parallel G x E trial with randomised complete block design arranged as a split plot factor with six replicates of the N treatments (1) L-arginine, (2) control and (3) NH_4NO_3 applied to main-plots. (b) Schematic representation of common block layout. Each block contained three main-plots, with ten genotypes as sub-plot factor, and 7 replicates from each genotype planted following row lines. In 2015, 2 years after the tree establishment, rhizosphere soil of 3 to 4 random replicates was collected from two contrasting genotypes (genotypes 31 and 48).

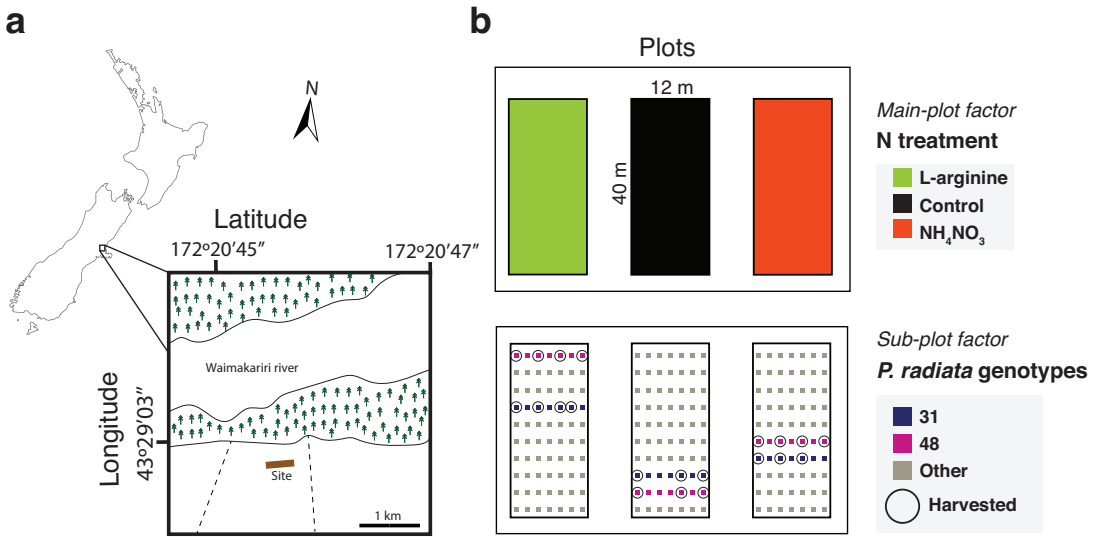

Figure C.2: Relationship between the ECM colonisation levels and soil moisture content. Points represent independent replicates coloured based on the N supply (control (black), L-arginine (green) and NH_4NO_3 (red)) and shape based on tree genotype (31 (round) and 48 (triangle)).

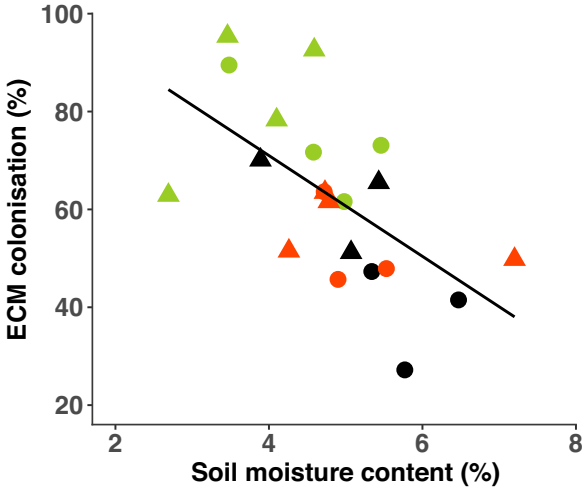

Table C.1: Effect of N treatment (N), tree genotype (Gen) and the interaction of both (G x N) in the observed OTU richness (Obs) and Shannon diversity index (H') across bacterial and fungal communities. *F*- statistics and *p*-values from two-way ANOVAs testing for effects of N treatment, tree genotype and their interaction (Gen x N) on soil chemistry are given. Bold values indicate statistically significant results, $p < 0.05$.

	Bacteria		**Rhizosphere fungi**		**Root-associated fungi**		**ECM fungi**	
	Obs	H'	Obs	H'	Obs	H'	Obs	H'
N	$F_{2,14}$=3.22	$F_{2,14}$=4.85	$F_{2,13}$=3.62	$F_{2,13}$=0.76	$F_{2,12}$=1.63	$F_{2,12}$=0.27	$F_{2,12}$=0.72	$F_{2,12}$=0.65
	p=0.071	***p*=0.025**	p=0.053	p=0.486	p=0.237	p=0.767	p=0.506	p=0.767
Gen	$F_{1,14}$=0.25	$F_{1,14}$=0.21	$F_{1,13}$=0.14	$F_{1,13}$=0.01	$F_{1,12}$=0.16	$F_{1,12}$=0.65	$F_{1,11}$=0.11	$F_{1,11}$=0.653
	p=0.624	p=0.652	p=0.714	p=0.963	p=0.699	p=0.435	p=0.742	p=0.434
GxN	$F_{2,14}$=4.11	$F_{2,14}$=8.91	$F_{2,13}$=0.54	$F_{2,13}$=0.47	$F_{2,12}$=0.38	$F_{2,12}$=0.38	$F_{2,12}$=0.11	$F_{2,12}$=0.38
	***p*=0.040**	***p*=0.003**	p=0.593	p=0.632	p=0.694	p=0.689	p=0.893	p=0.689

Table C.2: Permanova analysis using Bray-Curtis and Jaccard distances showing the influence of N treatment, tree genotype and the interaction of both (G x N) in explaining overall variance in microbial communities. Numbers in bold values indicate statistically significant results ($p < 0.05$).

		N treatment		**Genotype**		**G x N**	
		$\mathbf{F}_{(1,12)}$	p	$\mathbf{F}_{(2,12)}$	p	$\mathbf{F}_{(2,12)}$	p
Bacteria	Bray	2.13	**0.003**	1.79	**0.039**	1.46	0.057
	Jaccard	1.67	**0.003**	1.15	0.204	1.49	**0.008**
Rhizosphere fungi	Bray	1.64	0.070	0.98	0.430	1.07	0.351
	Jaccard	1.54	**0.014**	1.10	0.288	0.95	0.543
Root-associated fungi	Bray	0.76	0.807	1.48	0.124	0.76	0.791
	Jaccard	0.91	0.572	0.76	0.784	0.80	0.799
ECM root fungi	Bray	1.07	0.312	1.62	**0.023**	0.63	0.987
	Jaccard	0.52	0.827	0.73	0.586	0.96	0.497

L